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PRINCIPAL INVESTIGATOR: Jacqueline Moreno, Ph.D.

CONTRACTING ORGANIZATION: Leland Stanford Junior University
Stanford, California 94305-4125

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Jacqueline Moreno, Ph.D.

E-Mail: jmoreno1@stanford.edu

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Abstract

1,25-dihydroxyvitamin D3 (calcitriol), the active form of vitamin D3 is a hormone with anti-proliferative and pro-differentiation effects in prostate cancer (PCa) cells. Our ultimate goal is to identify novel therapeutic targets for the treatment of PCa with calcitriol. Previous to this study, we have used cDNA microarray analysis of established human PCa cell lines identifying, among others, the regulation of genes implicated in prostaglandin (PG) bioactivity. PGs are implicated in the development and progression of PCa, tumor invasiveness and tumor grade. We found that calcitriol down-regulates the expression of cyclooxygenase-2 (COX-2), enzyme that initiates PG synthesis, and up-regulates the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) involved in the first step in PG inactivation. In this project we analyze these two novel target genes of calcitriol. In this project we found that calcitriol reduces the expression of COX-2 mRNA and protein and up-regulates 15-PGDH mRNA and protein. We also found that the combined treatment of LNCaP and PC-3 cells with calcitriol and COX inhibitors mediate synergistic growth inhibition, allowing the use of reduced doses of both drugs that still resulted in enhanced anti-proliferative activity. The actions of calcitriol to reduce COX-2 expression and to induce 15-PGDH availability would potentially constitute a pathway to reduce and/or remove active PGs thereby diminishing PCa proliferation. These findings suggest that therapy combining calcitriol and COX-2 inhibitors will increase efficacy while decreasing side-effects. We strongly believe that the major contribution and significance of this project is to pave the way in the designing of new therapeutic approaches for PCa. We propose that the combination of these already approved drugs can be brought to a clinical trial swiftly. This study also helps in the understanding of the mechanisms of calcitriol action in prostate cells, generating new data to potentially aid in developing treatment strategies to improve PCa therapy. The ability of calcitriol to inhibit PG synthesis and stimulate PG destruction appears to be an additional pathway by which calcitriol can enhance PCa therapy. Based on these observations a clinical trial has recently been initiated by Dr. D. Feldman and Dr. S. Srinivas at Stanford University combining high doses of calcitriol and naproxen in PCa patients with advanced androgen-independent disease who have failed other therapies. The initial results are promising (Srinivas et al, unpublished observations).

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INTRODUCTION

Calcitriol, the hormonally active form of vitamin D, inhibits the growth and development of several cancers (*1-9*). There are multiple mechanisms underlying the anti-proliferative effects of calcitriol, which vary between target cells (*7, 9*). These include cell cycle arrest (*7, 9*) and the induction of apoptosis (*7*). Several genes that mediate these growth regulatory effects have been identified to be the molecular targets of calcitriol action, such as *p21*, *p27*, *bcl-2*, and *insulin-like growth factor binding protein-3* (*IGFBP-3*). We recently did cDNA microarray analyses to more fully characterize the spectrum of genes regulated by calcitriol in prostate cells (*10, 11*). Among the newly identified genes regulated by calcitriol, we found two genes which play a key role in prostaglandin (PG) metabolism: the prostaglandin endoperoxide synthase-2 or cyclooxygenase (COX)-2 and the NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). PGs are synthesized from free arachidonic acid (*12*) by COXs. There are two well-characterized COX isoforms: COX-1, a constitutive form of the enzyme, and COX-2, an inducible form of the enzyme. PGs are implicated in the initiation and progression of many malignancies including prostate cancer (*13-15*). Tumor cells with elevated COX-2 levels are highly resistant to apoptosis, show increased angiogenic potential, and exert suppressive effects on host immunity (*13-16*). Nonsteroidal anti-inflammatory drugs (NSAIDs), known inhibitors of both COX-1 and COX-2 enzymatic activity, are under intense investigation to prevent and/or treat malignancies (*17, 18*). 15-PGDH, which mediates the catalytic inactivation of PGs by converting them to the corresponding keto derivatives (*19*), has been found to be down-regulated in some cancers (*20, 21*) and has recently been regarded as a tumor suppressor gene (*20*).

Our hypothesis is that calcitriol regulation of PG metabolism (induction of 15-PGDH and inhibition of COX-2) is another pathway to remove active PGs that may help to diminish PCa proliferation. In this way, calcitriol and NSAIDs would have the same ultimate effect. Unfortunately, NSAID use has shown some secondary side-effects including increased risk of heart attacks, stroke, sudden death, blood clots, stomach and intestinal bleeding, kidney problems including acute kidney failure and worsening of chronic kidney failure (*22*). On the other hand, calcitriol also has secondary effects, namely hypercalcemia. However, this effect can be diminished with intermittent administration of calcitriol (*23*) or with the use of the new analogs of calcitriol (*12*). To avoid such unwanted actions we would resort to the combination of NSAIDs and calcitriol. We predict that the combination therapy could allow the use of lower doses of both drugs thus reducing their individual side-effects.

Given the induction of 15-PGDH expression and the inhibition of COX-2 expression by calcitriol, both NSAIDs and calcitriol would have the same effect, to reduce the pool of active PGs, but by different mechanisms. To us, this strongly suggests that induction of the regulation of PG-related metabolic genes by calcitriol contributes to its anti-proliferative activity. It also suggests a possible synergistic action of calcitriol and NSAID treatment to prevent cancer cell proliferation. We hypothesize that the role of calcitriol in the general metabolism of these eicosanoids, by induction of

15-PGDH and the inhibition of COX-2 expression, contributes to the anti-PCa action of calcitriol. By inhibiting COX-2 and stimulating 15-PGDH expression, calcitriol would decrease the levels of biologically active PGs in PCa cells and thereby reduce the proliferative stimulation of PGs, much like the NSAIDs. Our finding that calcitriol stimulates the expression of 15-PGDH and inhibits COX-2 assume greater significance considering the putative actions of PGs on PCa cells.

BODY

This study was directed to gain insight on the potential use of calcitriol, the active form of vitamin D, as an anti-proliferative agent in PCa by interfering with PG metabolism and actions.

Our microarray data indicated that calcitriol increased the expression of 15-PGDH and significantly decreased the expression of COX-2 in LNCaP human PCa cells (*23*). We hypothesize that this dual action of calcitriol would reduce the levels of biologically active PGs in PCa cells, thereby decreasing the proliferative stimulus for PCa growth.

Our experiments showed that calcitriol inhibits prostaglandin actions in PCa cells by three mechanisms: stimulating the expression of prostaglandin catabolizing enzyme 15-PGDH, decreasing the expression of prostaglandin synthesizing enzyme COX-2 and inhibiting EP2 and FP prostaglandin receptor expression [Moreno, 2005 #89; Moreno, 2005 #45]. We also observed a decrease in levels of PGs in the conditioned media of LNCaP cells treated calcitriol. We suspect that this was the result of the dual effect of calcitriol on the expression of PG metabolic enzymes (*24*). Furthermore, the combination of calcitriol and various non-steroidal anti-inflammatory drugs (NSAIDs) produced synergistic inhibition of PCa cell growth at 2 to 10 lower concentrations of the drugs needed to achieve the same effect alone. The findings suggest that calcitriol and NSAIDs may be a useful combination for chemotherapy PCa (*24*).

MATERIALS AND METHODS

Cell culture.

LNCaP (ATCC no. CRL-1740) and PC-3 (ATCC no. CRL-1435) cells were grown in RPMI 1640 supplemented with 5% FBS, 100 IU/mL of penicillin, and 100 μg/mL streptomycin (Life Technologies). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator.

Plasmid Constructs

The COX-2 cDNA was a kind gift from Dr. Stephen Prescott from the University of Utah. The construction pCOX-2 was obtained by amplifying the complete cDNA decoding the COX-2 by PCR using oligonucleotides: 5'-TTT TCT AGA ATG CTC GCC CGC GCC-3' and 5'-TTT GAT ATC CTA CAG TTC AGT CGA ACG-3'. The PCR product obtained with the *Taq* PCR Master Mix Kit (Qiagen Inc. Valencia, CA) was cloned into the pcR2.1 vector (Invitrogen, Carlsbad, CA). The COX-2 full length cDNA was isolated by *Eco* RI restriction and this fragment was sub-cloned into the *Eco* RI site was of the pcDNA3.1(-) eukaryotic expression vector (Invitrogen). The 15-PGDH expression vector cloned into the eukaryotic expression vector pcDNA1 (Invitrogen) was a kind gift from Dr. H. H. Tai, from the University of Kentucky.

Transfection

Plasmids were purified with the Qiagen plasmid purification kit according to the manufacturer's instructions. Subconfluent PC-3 or LNCaP cells were transfected using Trans IT Prostate (Mirus Bio Corporation. Madison, WI.) according to the manufacturer's instructions. For stable expression, cells were screened first for their resistance to 600 μg/ml of G418 (Geneticin, Invitrogen) and then for either COX-2 or 15-PGDH over-expression using Western blot analysis and

measuring PG content in the conditioned media of transfected cells. Cells were maintained in medium containing 200 μg/ml of G418.

Small interfering RNA transfection.

Subconfluent LNCaP cells were transfected with 30 nmol/L of negative control (Ambion, Austin, TX), or the *Silencer* Pre-designed 15-PGDH-directed siRNA (# 16708) siRNA (Ambion) using siPORT NeoFX (Ambion). Cells were used for experiments Western blot analysis after transfection as indicated.

RNA isolation and real-time reverse transcription-PCR. These procedures were carried out exactly as described in (*1*) (please see Appendix II). Table I lists the primers used. Briefly, total RNA was extracted from cells using Trizol reagent (Invitrogen). Five μg of total RNA were used to obtain first strand cDNA using the SuperScript III kit (Invitrogen). Gene expression in vehicle or calcitriol-treated cells was determined by real-time PCR using the reverse transcription product and gene-specific primers (10 pmol) with the DyNamo SYBR Green qPCR kit (Finnzymes, Oy, M.J. Research). Real-time PCR was carried out using an Opticon 2 DNA engine (M.J. Research). Relative changes in mRNA expression levels were assessed by the $2^{-\Delta\Delta C(T)}$ method (*2*). Changes in mRNA expression of the different genes were normalized to that of TATA binding protein (*TBP*) gene.

TABLE 1. Primers used in real-time RT-PCR.

Gene	Forward	Reverse
bcl-2	5′-TGGGATGCCTTTGTGGAACTAT-3′	5′-GAGACAGCCAGGAGAAATCAAAC-3’
Bax	5'-AGGG TTTCATCCAGGATCGAGCAG-3'	5'-ATCTTCTTCCAGATGGTGAGCGAG-3'
Cyclin D	5’-CCGTCCATGCGGAAGATC-3’	5’-ATGGCCAGCGGGAAGAC-3’
p21	5′-GCAGACCAGCATGACAGATTT-3′	5′-GGATTAGGGCTTCCTCTTGGA-3′
IGFBP-3	5’-AAGTTCCACCCCCTCCATTC-3’	5’-TCTTCCATTTCTCTACGGCAG-3’
AR	5’-AGTCCCACTTGTGTCAAAAGC-3’	5’-ACTTCTGTTTCCCTTCAGCG-3’
VDR	5’-CACTGGCTTTCACTTCAATGC-3’	5’-CGATGTCCACACAGCGTTTG-3’
TGF-b	5’-GATTTCCATCTACAAGACCACGAGG-3’	5’-GCATCAGTTACATCGAAGGAGAGC-3’
VEGF	5’-GAGGAGTCCAACATCACCATGC-3’	5’-CGTTTAACTCAAGCTGCCTCGCC-3’
COX-2	5'-GATACTCAGGCAGAGATGATCTACCC-3'	5'-AGACCAGGCACCAGACCAAAGA-3'
15-PGDH	5'-GACTCTGTTCATCCAGTGCG-3'	5'-CCTTCACCTCCATTTTGCTTACTC-3'
TBP	5'-CACTCACAGACTCTCACAACTGC-3'	5'-GTGGTTCGTGGCTCTCTTATC-3'

RESULTS

Calcitriol exhibits anti-proliferative and pro-differentiation effects in prostate cancer. Our goal in this project was to contribute in further defining the mechanisms underlying these actions in human PCa cell lines.

This project was divided into two parts. In the first part we found that calcitriol significantly reduced the mRNA and protein expression of COX-2, the key PG synthesis enzyme. Calcitriol also up-regulated the expression of 15-PGDH, the enzyme initiating PG catabolism. This dual action was associated with decreased PGE_2 secretion into the conditioned media of PCa cells exposed to calcitriol. Calcitriol also repressed the mRNA expression of the PG receptors EP2 and FP, providing a potential additional mechanism of suppression of the biological activity of PGs. Importantly, calcitriol attenuated PG-mediated prostate cancer cell growth and blocked the induction of some of the functional targets of PGs, like *c-fos* mRNA expression. The combination of calcitriol with

nonsteroidal anti-inflammatory drugs (NSAIDs) synergistically acted to achieve significant PCa cell growth inhibition at ~2 to 10 times lower concentrations of the drugs than when used alone. These results were published in Cancer Research (*1*). Please see the full length paper in Appendix II for further details.

In the second part of this project our goal was to gain an insight on how the combination of calcitriol and NSAIDs might improve the growth inhibitory actions of each drug alone and to asses the relative contribution of COX-2 and 15-PGDH regulated expression on the growth inhibitory actions of calcitriol.

Potential Mediators of the Enhanced Growth Inhibition by the combined treatment with Calcitriol and Naproxen in PCa cells

The combined treatment of LNCaP and PC-3 cells with calcitriol and NSAIDs results in the synergistic inhibition of cell growth, as indicated in Figure 5 in Moreno et al. (*1*) (Please see Appendix II). This is true for both COX-2-specific NSAIDs or COXIBs (SC-5825 and NS398) and non-specific NSAIDs (naproxen and ibuprofen). To explore the possible molecular mechanisms mediating this enhanced growth inhibition, we analyzed the effects of the combination of calcitriol and naproxen, a non-specific NSAID For this analysis we used non-specific NSAIDs. This decision was based on the equal effectiveness of both classes of NSAIDs to inhibit growth when combined with calcitriol and on the recent safety concerns about the chronic use of COXIBs (*1*). We analyzed apoptosis-related genes (*bcl-2*, *bax*), cell cycle-related genes (*Cyclin D1*, *p21*, *IGFBP-3*), PG metabolism-related genes (*COX-2* and *15-PGDH*), steroid receptor genes (*AR* and *VDR*) and growth factor genes (*VEGF*, *TGF-β*) in LNCaP cells. As shown in Table II, the expression of *bax*, *Cyclin D1*, *p21*, *COX-2*,*15-PGDH*, *AR*, *VDR*, and *TGF-β* mRNAs in LNCaP cells is regulated by either calcitriol 10 nM or naproxen 150 μM alone, however, the combined treatment of LNCaP cells with the two drugs has no further effect on the mRNA expression levels of these genes (Table II).

Table II. Real-time RT-PCR analysis of gene expression in response to the combination of calcitriol and two doses of naproxen. Mean ± SD.

	Vehicle	Calcitriol 10 nM	Naproxen 150 uM	Naproxen 150 uM
Apoptosis				
bcl-2	1.15 ± 0.15	0.61 ± 0.02	0.42 ± 0.06	0.21 ± 0.03
Bax	1.09 ± 0.29	0.55 ± 0.14	0.32 ± 0.05	0.41 ± 0.04
Cell-cycle				
Cyclin D1	0.95 ± 0.04	0.48 ± 0.16	0.32 ± 0.05	0.32 ± 0.01
IGFBP-3	1.01 ± 0.11	4.54 ± 0.48	2.94 ± 0.08	6.99 ± 0.45
p21	1.14 ± 0.27	0.96 ± 0.11	3.69 ± 0.55	3.73 ± 0.41
PG-metabolism				
COX-2	1 .67 ± 0.27	0.67± 0.12	0.66 ± 0.13	0.68 ± 0.47
15-PGDH	1 ± 0.07	3.12 ± 0.45	2.37 ± 0.79	3.69 ± 0.56
Nuclear Receptors				
AR	1.47 ± 0.03	1.81 ± 0.22	1.15 ± 0.01	1.74 ± 0.17
VDR	0.88 ± 0.43	0.48 ± 0.07	0.61 ± 0.09	0.74 ± 0.31
Growth factors				
VEGF	0.78 ± 0.21	1.15 ± 0.45	0.62 ± 0.11	0.34 ± 0.09
TGF-beta	1.44 ± 0.12	0.85 ± 0.21	0.49 ± 0.06	0.41 ± 0.29

In the case of *bcl-2*, *IGFBP-3*, and *VEGF* mRNA expression, the combination of calcitriol and naproxen potentiated the effects of each drug alone. The expression of *bcl-2* mRNA was down-regulated by both calcitriol and naproxen acting alone (**Figure 1A**), however, the combination resulted in the synergistic decrease of the expression of *bcl-2* (**Figure 1A**). The same effect was observed in the expression of *VEGF* mRNA (**Figure 1C**). *IGFBP-3* mRNA expression is induced by calcitriol 10 nM and modestly increased by naproxen 150 μM (**Figure 1B**), with the combination of calcitriol inducing a further increase. This suggests that the combination of naproxen and calcitriol may act through the induction of apoptosis (*2*) and attenuating the actions of growth factors (*3*). The combined effect of calcitriol and naproxen in decreasing the mRNA levels of *VEGF* results is of particular interest, considering its role in angiogenesis in PCa (*4*).

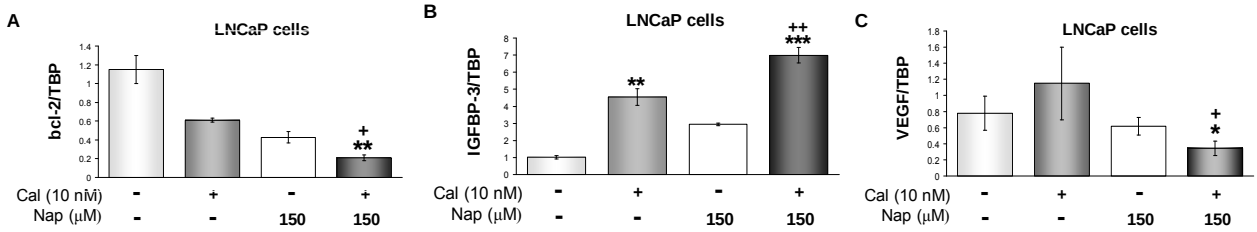

Figure 1. Synergistic regulation of gene mRNA expression in LNCaP cells by calcitriol and naproxen. Subconfluent LNCaP cells were treated with 0.1% ethanol vehicle (-) or 10 nmol/L calcitriol (Cal) in the presence and absence of the indicated concentration of 150 μmol/L naproxen (Nap) for 24 hours. Total RNA was extracted and analyzed for the mRNA expression of *bcl-2* (A), *IGFBP-3* (B) or VEGF (C) by real-time RT-PCR using gene-specific primers. Gene mRNA levels were normalized to *TBP* mRNA levels. Values given as a fraction of control set at 1; columns, mean from two experiments; bars, SD. *. P < 0.05, **, P < 0.01,***, P < 0.001 when compared with control. +, P < 0.05, ++, P < 0.01 when compared to naproxen alone.

To expand our knowledge on the molecular pathways mediating the enhanced effect of the combination of calcitriol and NSAIDs on PCa cell growth inhibition, we plan to use pathway-focused arrays, which are designed to determine the expression profile of a pathway-specific panel of genes.

We are currently studying the effects of the calcitriol naproxen combination on the leves of secreted PGs to the conditioned media.

Role of 15-PGDH increased expression and the COX-2 reduced expression in the anti-proliferative actions of calcitriol

We have previously shown that calcitriol increases the expression of 15-PGDH mRNA and protein (*1*). 15-PGDH has a role in mediating growth inhibition (*3*). Our hypothesis is that the calcitriol-induced increase in 15-PGDH expression also plays a role in the growth inhibitory actions of calcitriol. To study this possibility, we decided to knock-down the expression of 15-PGHD and measure the effects of this knock-down on the growth inhibition induced by calcitriol in LNCaP cells. We transfected LNCaP cells with a siRNA directed against 15-PGDH and separately with a control siRNA for comparison. We first analyzed the effects of the siRNA transfection on the 15-PGDH protein expression in Western blot assays. We found that the siRNA directed against 15-PGDH effectively decreases the expression of the15-PGDH protein (lane 2, **Figure 2A**) when compared to the levels observed in cells transfected with the control siRNA (lane 1, **Figure 2A**). However, these effects are reversed at 72 h (lane 3, **Figure 2A**), when the 15-PGDH protein levels seem to increase again.

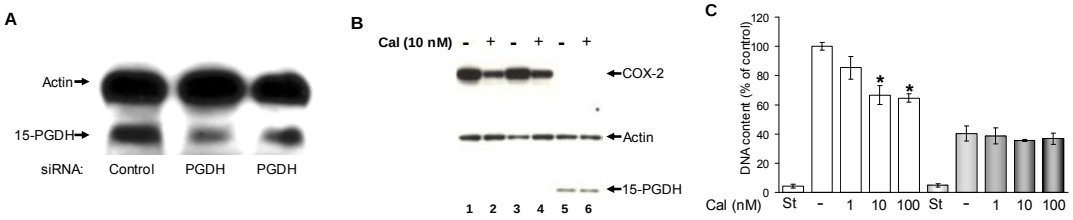

Figure 2. Role of 15-PGDH in calcitriol-mediated growth inhibition in PCa cell lines. *A*, 15-PGDH down-regulation by siRNA in LNCaP cells. Representative Western blot orLNCaP cells transfected with either a control (Control) or a 15-PGDH-directed siRNA (PGDH). Protein extracts were obtained at 48 (lane 2) or 72 h (lane 3). The expression levels of 15-PGDH were determined by Western blot analysis. Actin detection was included as a loading control. *B* 15-PGDH stable transfection in PC-3 cells. Two independent clones of PC-3 cells transfected with the construct pcDNA1-15-PGDH (PGDH, lanes 3 to 6) cDNA or with the empty vector (Control, lanes 1 and 2), were treated with 10 nMol/L calcitriol for 48 h, harvested and 15-PGDH and COX-2 levels were determined by Western blot. Actin immunodetection was used as loading control. *C*, effects of 15-PGDH over-expression on the growth inhibitory actions of calcitriol in PC-3 cells stably transfected with pcDNA1 vector (empty columns) or PGDH construct.(gray columns) PC-3 cells were treated with 0.1% ethanol vehicle (-) or the indicated concentration of calcitriol (Cal). St indicated the levels of DNA at the beginning of the assay. Cell growth was determined by measuring the DNA content. DNA contents are given as percentage of control pcDNA transfected cell value set at 100%. 100% DNA content for control pcDNA1 cells = 39.12 ± 6.21 μg/well; 100% DNA content for PGDH10 PC-3 cells = 22.48 ± 9.66 μg/well. Columns, mean from two experiments; bars, SD. *, $P < 0.05$ when compared with control.

The effects of 15-PGDH on the growth inhibitory effects of the calcitriol can be alternatively studied by over-expressing the protein in 15-PGDH-negative cells. We decide stably transfected PC-3 cells with the 15-PGDH cDNA, since PC-3 cells lack detectable levels of 15-PGDH (*1*). After the transfection, G418-resistant cells were screened for 15-PGDH expression by immuno-detection of the protein using Western blots and by measuring the levels of secreted PGE_2 in the conditioned media of transfected cells (data not shown). We obtained two independent clones of PC-3 cells expressing 15-PGDH. One of them expressed high levels of the active enzyme as indicated by a 10-fold reduction on the secreted PGE_2 levels. Interestingly, we were no longer able to detect COX-2 protein expression in these cells using Western blot (lanes 5 and 6, **Figure 1B**) In contrast, the protein was detected in the control cells (pcDNA1-transfected cells, (lanes 1 and 2, **Figure 2B**) and in the low (with 2-fold reduction in PGE_2 levels respect to the pcDNA1 transfected cells) 15-PGDH expressing clones s (lanes 3 and 4, **Figure 2B**). We next examined the effects of the 15-PGDH over-expression on the growth inhibitory actions of calcitriol in PC-3 cells transfected with the empty pcDNA1 vector or the high 15-PGDH expressing clone. The starting levels of DNA (St columns, **Figure 2C**) were comparatively the same in both pcDNA1 cells or in PGDH cells. At the end of the six-day treatment, the DNA levels in the control of pcDNA1 cells was notably higher (39.12 ± 6.21 μg DNA/well) than in those cells over-expressing 15-PGDH (22.48 ± 9.66 μg DNA/well). In the pcDNA1-transfected cells (empty columns, **Figure 2C**), calcitriol had a modest effect (~20%) at 1 nmol/L but caused significant growth inhibition (~40%) at 10 nmol/L. These effect, however, is lost in those cells over-expressing 15-PGDH (gray colums, Figure 2C). As indicated, the15-PGDH expressing PC-3 cells we used have a comparatively reduced levels of COX-2 expression and markedly reduced levels of PGE_2. Also, we found a comparatively lower rate of growth in these cells when campared to the empty vector transfected cells. This already slow proliferative state may limit the effects of calcitriol. We believe that this unforeseen aspect of the approach can be salved by using inducible

expression systems to direct the levels and time of 15-PGDH expression, allowing the cells to develope normally until treated with calcitriol.

We previously found that calcitriol significantly reduced the mRNA and protein expression of COX-2 (*1*). We hypothesize that the decrease on COX-2 expression by calcitriol has a role on the growth inhibitory actions of the hormone. Our approach was to over-express COX-2 in PCa cells and then evaluate the effects of this over-expression on the ability of calcitriol to inhibit the growth of these cells. We first cloned the full-length COX-2 cDNA in the pcDNA3.1(-) expression plasmid (see Methods). We stably transfected PC-3 with these plasmids. After the selection with G418, we screened for COX-2 over expression in Western blot assays. We obtained two independent clones of PC-3 cells over-expressing COX-2 (**Figure 3C**). We will use these cells to evaluate the effects of this over-expression on the ability of calcitriol to inhibit their growth.

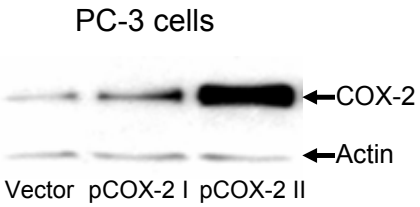

Figure 3. COX-2 over-expression in PC-3 cells. PC-3 cells were stably transfected with the plasmid construct pCOX-2. *A*, COX-2 overexpression was assessed by Western blot analysis. Actin immuno-detection was used as loading control.

KEY RESEARCH ACCOMPLISHMENTS

Training accomplishments

Dr. Moreno has spent her time performing experiments in the laboratory of Dr. David Feldman, under the guidance of Dr. Feldman and Dr. Aruna Krishnan, a very experienced Research Scientist.

Dr. Moreno has periodically presented her data to the group of collaborators from the laboratories of Dr. Feldman and Dr. Donna Peehl, a long time collaborator, at the weekly lab meetings. Dr. Moreno has made formal presentations to the Stanford Prostate Cancer Group and to the Department of Urology Research Conference.

Dr. Moreno attended classes in "Responsible Conduct of Research", once a week for eight consecutive weeks in the period of September-November, 2004. The course was comprised of lectures on environmental health and safety, use and protection of human subjects and lab animals, conflicts of interest, publication, intellectual property and data, error, negligence or misconduct and response to violations of ethical standards. She also received a lecture on "How to Write a Grant" on December 2005. This lecture sponsored by the Office of Postdoctoral Affairs is a training workshop was dedicated to examine the mechanisms of writing and assembling an NIH grant. During the period of January to April 2006, Dr. Moreno attended the series of Scientific Management, developed by the by the Office of Postdoctoral Affairs and designed to provide with laboratory and research management skills to launch a productive independent career.

Dr. Moreno has also gained experience in prostate cancer biology, normal and abnormal prostate cell function, prostate cancer therapy, chemoprevention strategies and the design of new treatment therapies to delay or prevent prostate cancer progression. The methods employed focus on hormone action, nuclear receptors, and regulation of target gene transcription and protein expression as well as metabolic studies of enzymatic activity and regulation of gene product concentration. The methods applied included cell culture, gene expression analysis by real time RT-PCR, Western blot, gene regulation studies, transfection and reporter gene assays.

During the period of September 2004 to September 2006, Dr. Moreno completed the publication of six papers, three of them directly related to this fellowship published in the *Journal of Steroid Biochemistry and Molecular Biology*, *Cancer Research* and *Anticancer Research* (see Appendix I-Biosketch). The results of the current project have also been presented at several meetings: (1) by Dr. Feldman at the 10th Prouts Neck Meeting on Prostate Cancer, in Maine in October 2004, (2) at the Vitamin D Symposium on Cancer, organized by the NIH: Chemoprevention & Cancer Treatment: Is there a role for vitamin D, 1α,25(OH)2-vitamin D3 or new analogs (deltanoids)", in November, 2004 in Bethesda, Maryland, (3) at the 2nd Int. Symposium on Vitamin D Analogs in Cancer Prevention and Therapy. May 2005, Lübeck, Germany, (4) by Dr. Moreno at the Endocrine Society's 87th Annual Meeting, in San Diego California in June 2005 and (5) at the Endocrine Society's 88th Annual Meeting, in Boston Massachusetts in June 2006.

Research accomplishments

The major part of the scientific accomplishments is described in detail in the paper published in Cancer Research (Vol. 65: 7917, Sept. 2005), found in the Appendix.

Calcitriol exhibits anti-proliferative and pro-differentiation effects in prostate cancer. Our goal is to further define the mechanisms underlying these actions. We studied established human prostate cancer cell lines and showed that calcitriol interferes with the metabolism of prostaglandins (PGs), known stimulators of prostate cell growth in three ways:

- Calcitriol significantly repressed the mRNA and protein expression of prostaglandin endoperoxide synthase/cyclooxygenase-2 (COX-2), the key PG synthesis enzyme.
- Calcitriol up-regulated the expression of 15-hydroxyprostaglandin dehydrogenase, the enzyme initiating PG catabolism.
- We found that this dual action was associated with decreased prostaglandin E2 secretion into the conditioned media of prostate cancer cells exposed to calcitriol.
- Calcitriol also repressed the mRNA expression of the PG receptors EP2 and FP, providing a potential additional mechanism of suppression of the biological activity of PGs.
- Calcitriol treatment attenuated PG-mediated functional responses, including the stimulation of prostate cancer cell growth and the up-regulation of PG target genes as *c-fos*.
- The combination of calcitriol with NSAIDs synergistically acted to achieve significant prostate cancer cell growth inhibition at approximately 2 to 10 times lower concentrations of the drugs than when used alone.
- The regulation of PG metabolism and biological actions constitutes a novel pathway of calcitriol action that may contribute to its anti-proliferative effects in prostate cells.
- We propose that a combination of calcitriol and nonselective NSAIDs might be a useful chemo-preventive and/or therapeutic strategy in men with prostate cancer, as it would allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects.
- Based on these observations we have recently initiated a clinical trial of calcitriol and naproxen combination in PCa patients with advanced androgen-independent disease who have failed other therapies. The regimen includes very high doses of calcitriol (45 micrograms of DN-101 from Novacea) once weekly combined with naproxen (400 mg, twice daily) and the initial results are promising (Srinivas et al, unpublished observations).

REPORTABLE OUTCOMES

Publications:

- **Moreno J.**, Krishnan A.V., Swami S., Nonn L., Peehl D.M., Feldman, D. 2005. Regulation of prostaglandin metabolism by calcitriol attenuates growth stimulation in prostate cancer cells. *Cancer Res.* **65**: 7917-7925.
- **Moreno, J.**, Krishnan, A.V., Feldman, D. 2005. Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer. *J. Steroid. Biochem. Mol. Biol*. **97**: 31-36.
- **Moreno, J.**, Krishnan, A.V., Feldman, D. 2006. Mechanisms of vitamin D-mediated growth inhibition in prostate cancer cells: inhibition of the prostaglandin pathway. *Anticancer Res*. **26**(4A): 2525-2530.
- Krishnan AV, Moreno J, Nonn L, Swami S, Peehl DM and Feldman D. Calcitriol as a Chemopreventive and Therapeutic Agent in Prostate Cancer: Role of Anti-inflammatory Activity. *J. Bone Mineral. Res.* In press.

Scientific Meetings:

- Feldman, D., **Moreno, J**., Krishnan A.V. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. 10th Prouts Neck Meeting on Prostate Cancer, Prouts Neck, Maine October 2004,
- Feldman, D., **Moreno, J**., Krishnan A.V. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. Vitamin D symposium: Cancer Chemoprevention & Cancer Treatment: Is there a role for vitamin D, 1α,25(OH)2-vitamin D3 or new analogs (deltanoids)" November, 2004, Bethesda, Maryland.
- **Moreno, J**., Krishnan, A.V., Feldman, D. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. Abstracts of the 2nd Int. Symposium on Vitamin D Analogs in Cancer Prevention and Therapy. May 2005, Lübeck, Germany. *Anticancer Res.* 25. 2290.
- **Moreno J**., Krishnan AV, Feldman D. Regulation of prostaglandin metabolism by calcitriol: Potential role in the treatment of prostate cancer. Endocrine Society's 87th Annual Meeting, in San Diego California, June 2005.
- **Moreno J**., Krishnan AV, Peng L, Swami S, Johnson C, Feldman D. Regulation of prostaglandin metabolism by calcitriol. Endocrine Society's 88th Annual Meeting, in Boston Massachusetts, June 2006.

Clinical Trial.

Our findings have now been translated to a clinical trial in men with advanced, androgen-independent prostate cancer who have failed other therapies. The trial is being carried out by Dr. Sandy Srinivas, a medical oncologist who runs the urology cancer section at Stanford University School of Medicine with the assistance of Dr. Feldman. The protocol includes DN 101 (Novacea) 45

micrograms once weekly and naproxen 400 mgms twice daily. At this time 12 patients have been entered into the trial and accrual is continuing. A majority of the pateints have shown a prolongation of the PSA doubling time indicating a slowing of the prostate cancer growth. Considering that these men have failed all other therapies, this finding is very encouraging of a unique benefit of this therapeutic regimen.

CONCLUSIONS

Our research is directed at understanding the molecular mechanisms of the anti-proliferative activity of calcitriol in prostate cells with the goal of developing strategies to improve PCa treatment. Using cDNA microarrays we have recently found that calcitriol modulates the expression genes involved in PG metabolism. Calcitriol reduces the expression of COX-2 gene, the enzyme that catalyzes PG synthesis and up-regulates the expression of 15-PGDH gene, the enzyme involved in PG inactivation. In the current project we found that calcitriol acts by three separate mechanisms: decreasing COX-2 expression, increasing 15-PGDH expression, and reducing PG receptor mRNA levels. We believe that these actions contribute to suppress the proliferative stimulus provided by PGs in prostate cancer cells. Calcitriol treatment attenuated PG-mediated functional responses, including the stimulation of prostate cancer cell growth, the secretion of PGs to the conditioned media of PCa cells treated with calcitriol and blocking the up-regulation of PG target genes. The combination of calcitriol with nonsteroidal anti-inflammatory drugs (NSAIDs), known inhibitors of the enzymatic activity of COX-2, synergistically acted to achieve significant prostate cancer cell growth inhibition at ~2 to 10 times lower concentrations of the drugs than when used alone. The regulation of PG metabolism and biological actions constitutes an additional novel pathway of calcitriol action mediating its anti-proliferative effects in prostate cells. We propose that a combination of calcitriol and a nonselective NSAID, such as naproxen, might be a useful therapeutic and/or chemo-preventive strategy in prostate cancer, as it would achieve greater efficacy and allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects. The action of calcitriol at the genomic level to suppress COX-2 gene expression will decrease the levels of COX-2 protein and allow the use of lower the concentrations of NSAIDs needed to inhibit COX-2 enzyme activity. The most significant contribution of the present project was the establishment of a rationale for the treatment advanced PCa. Recently Drs. S. Srinivas and D. Feldman at Stanford University have initiated a clinical trial treating patients with advanced androgen-independent disease who have failed other therapies with a combination of calcitriol and naproxen. The regimen includes very high doses of calcitriol (45 micrograms of DN-101 from Novacea) once weekly combined with naproxen (400 mg, twice daily). The initial results indicate a reduction of the PSA rise in some patients (Srinivas et al, unpublished observations).

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APPENDIX I

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Jacqueline Moreno	Postdoctoral fellow

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Center for Research and Advanced Studies, Mexico City. MEXICO	M.S.	1998	Cell Biology
Center for Research and Advanced Studies, Mexico City. MEXICO	Ph.D.	2002	Cell Biology

Doctoral Research. July 1998- October 2002. Department of Physiology, Biophysics and Neurosciences, Center for Research and Advanced Studies. Research Advisor Dr. Marcelino Cereijido.

Molecular and cell biology of the epithelial transporting phenotype.

- Study of the sorting signals and mechanisms involved in membrane protein targeting.
- Analysis of protein-protein interaction motifs and their involvement in determining protein stability and retention.
- Role of tight junction associated protein phosphorylation on epithelial cell functional integrity.

Research Experience

Postdoctoral Associate. October 2002-June 2003, Center for Research and Advanced Studies. Study of the role of tight junction proteins and Na,K-ATPase β-subunit on epithelial transport phenotype genesis and maintenance.

Postdoctoral Fellow. November 2003- . Stanford University. Analysis of the mechanisms of 1α,25-DihydroxyVitamin-D3.

Scholarships

1993-1999. Fellow of the National Science and Technology Council (CONACyT, Mexico).

2004- Postdoctoral Traineeship Award. Department of Defense Prostate Cancer Research Program (PC04120).

Publications and Presentations

- Avila-Flores, A., Rendon-Huerta, E., **Moreno, J.**, Islas, S., Betanzos, A., Robles-Flores, M., Gonzalez-Mariscal, L. 2001. Tight-junction protein zonula occludens 2 is a target of phosphorylation by protein kinase C. *Biochem. J.* **360**: 295-304
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- Feldman, D., **Moreno, J**., Krishnan, A.V. 2004. Pathways mediating the growth inhibitory actions of vitamin D in prostate cancer. Abstract in the Workshop on Cancer Chemoprevention & Cancer Treatment: Is there a role for vitamin D, 1α,25$(OH)_2$-vitamin D_3 or new analogs (deltanoids)?. NIH, Bethesda, MD.
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- **Moreno, J**., Krishnan, A.V., Swami, S., Nonn, L., Peehl, D.M., Feldman, D. 2005. Regulation of prostaglandin metabolism by calcitriol attenuates growth stimulation in prostate cancer cells. *Cancer Res.* **65**: 7917-7925.
- **Moreno, J**., Krishnan, A.V., Peehl, D.M., Feldman, D. 2006. Mechanisms of vitamin D-mediated growth inhibition in prostate cancer cells: inhibition of the prostaglandin pathway. *Anticancer Res.* 2006; 26(4A): 2525-2530.

Scientific Meetings:

- Feldman, D., Moreno, J., Krishnan A.V. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. 10th Prouts Neck Meeting on Prostate Cancer, Prouts Neck, Maine October 2004,
- Feldman, D., Moreno, J., Krishnan A.V. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. Vitamin D symposium: Cancer Chemoprevention & Cancer Treatment: Is there a role for vitamin D, 1α,25(OH)2-vitamin D3 or new analogs (deltanoids)" November, 2004, Bethesda, Maryland.
- Moreno, J., Krishnan, A.V., Feldman, D. Pathways mediating the growth inhibitory actions of Vitamin D in prostate cancer. Abstracts of the 2nd Int. Symposium on Vitamin D Analogs in Cancer Prevention and Therapy. May 2005, Lübeck, Germany. *Anticancer Res.* 25. 2290.
- Moreno J., Krishnan A.V., Feldman D. Regulation of prostaglandin metabolism by calcitriol: Potential role in the treatment of prostate cancer. Endocrine Society's 87th Annual Meeting, in San Diego California, June 2005.
- Moreno J., Krishnan A.V., Peng L., Swami S., Johnson C., Feldman D. 2006. Regulation of the Prostaglandin Pathway by Calcitriol in Prostate Cancer. Endocrine Society's 88th Annual Meeting, in Boston Massachusetts, June 2006.

APPENDIX II

Publications relevant to this award:

2004-2005:

J. Steroid Biochem. Mol. Biol. 2005; 97(1-2): 31-36.

Cancer Res. **65**: 7917-7925.

2005-2006:

Anticancer Res. 2006; 26(4A): 2525-2530.

Scientific Meetings:

2004-2005:

- 10th Prouts Neck Meeting on Prostate Cancer, Prouts Neck, Maine October 2004,
- Abstracts of the 2nd Int. Symposium on Vitamin D Analogs in Cancer Prevention and Therapy. May 2005, Lübeck, Germany. *Anticancer Res.* 25. 2290.
- Endocrine Society's 87th Annual Meeting, in San Diego California, June 2005.

2005-2006:

- Endocrine Society's 88th Annual Meeting, in Boston Massachusetts, June 2006.

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Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer

Jacqueline Moreno, Aruna V. Krishnan, David Feldman *

Department of Medicine, Division of Endocrinology, Stanford University School of Medicine, Stanford, CA 94305, USA

Abstract

Calcitriol (1,25-dihydroxyvitamin D_3) inhibits the growth and stimulates the differentiation of prostate cancer (PCa) cells. The effects of calcitriol are varied, appear to be cell-specific and result in growth arrest and stimulation of apoptosis. Our goal was to define the genes involved in the multiple pathways mediating the anti-proliferative effects of calcitriol in PCa. We used cDNA microarray analysis to identify calcitriol target genes involved in these pathways in both LNCaP human PCa cells and primary prostatic epithelial cells. Interestingly, two of the target genes that we identified play key roles in the metabolism of prostaglandins (PGs), which are known stimulators of PCa cell growth and progression. The expression of the PG synthesizing cyclooxygenase-2 (*COX-2*) gene was significantly decreased by calcitriol, while that of PG inactivating 15-prostaglandin dehydrogenase gene (*15-PGDH*) was increased. We postulate that this dual action of calcitriol would reduce the levels of biologically active PGs in PCa cells decreasing their proliferative stimulus and contribute to the growth inhibitory actions of calcitriol. In addition, we propose that calcitriol can be combined with non-steroidal anti-inflammatory drugs that inhibit COX activity, as a potential therapeutic strategy to improve the potency and efficacy of both drugs in the treatment of PCa.

Keywords: CYP24; CYP27B1; VDR; cDNA arrays; Target genes; Prostaglandins; 15-PGDH; COX-2; NSAIDs

1. Introduction

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death in North American men. Primary therapy to treat PCa involves the surgical removal of the prostate or radiation therapy. However, in many men the cancer progresses to advanced or metastatic disease. Androgens play a crucial role in the development, growth and maintenance of the prostate. Most patients with metastatic PCa who have failed the primary therapy, receive drugs that block the production of androgens [1]. Although most men have a good initial response to the androgen deprivation therapy, almost all of them will eventually relapse after an average of 2–3 years. This progression develops when the cancer has evolved from androgen-dependent to androgen-independent PCa (AIPC) with limited treatment options and becomes ultimately lethal. 1,25-Dihydroxyvitamin D_3 (calcitriol), the active metabolite of Vitamin D, has emerged in recent years as a promising therapeutic agent in the treatment of PCa [2–11]. Calcitriol is an important regulator of calcium homeostasis and bone metabolism through its actions in intestine, bone, kidney and the parathyroid glands [12]. In addition to these classical actions, calcitriol also exerts anti-proliferative and pro-differentiating effects in a number of tumors and malignant cells including PCa raising the possibility of its use as an anti-cancer agent.

2. Calcitriol and prostate cancer

2.1. Epidemiological and genetic studies

PCa development has been shown to be associated with age, genetic factors and race [1]. Various studies indicate that dietary [13] and environmental factors also play a role in PCa genesis. Epidemiological data provide a strong correlation between the exposure to sunlight and the prevalence of certain cancers, particularly prostate cancer [14]. Since UV light

* Corresponding author. Tel.: +1 650 725 2910; fax: +1 650 725 7085.
E-mail address: feldman@cmgm.stanford.edu (D. Feldman).

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is essential for Vitamin D synthesis in the skin, these studies have led to the suggestion that low circulating levels of calcitriol increase the probability of developing PCa. Moreover, polymorphisms in the gene encoding the Vitamin D receptor (VDR), which mediates the biological activity of calcitriol, may be involved in the development and or progression of PCa [2–11,13,15].

2.2. Prostate as a target for calcitriol

Many studies have demonstrated a beneficial effect of calcitriol to inhibit PCa growth and progression [2–11]. Calcitriol exerts anti-proliferative actions in several PCa cell lines [16–18], as well as in primary cultures of normal and cancer cells derived from surgical specimens taken from men with PCa [19]. The inhibition of PCa cell growth is seen in both androgen-dependent and AIPC cells [20]. Several in vivo studies on tumor xenografts established by transplanting clinical prostate tumors or cultured human PCa cells into immune-deficient mice have also demonstrated the tumor inhibitory effects of calcitriol [2–11]. The concentrations of calcitriol required for eliciting a significant growth inhibitory response in vivo causes hypercalcemia as a side effect. Therefore, investigators have used structural analogs of calcitriol that exhibit reduced calcemic effects in in vivo studies [2–7,16]. Recent findings suggest that large doses of calcitriol can be safely given to patients if administered intermittently [8,10].

3. The role of calcitriol metabolism in cellular responsiveness to the hormone

3.1. 25-Hydroxyvitamin D_3-1-α-hydroxylase (CYP27B1)

25-Hydroxyvitamin D_3-1-α-hydroxylase (1α-hydroxylase or CYP27B1) is the enzyme mediating the conversion of 25(OH)D_3 to calcitriol in the kidney, which is the major site of calcitriol synthesis [12]. Interestingly, it has been found that 1α-hydroxylase is also expressed in tissues other than the kidney including the prostate [21]. This suggests that prostate tissue is able to produce calcitriol locally and raises the possibility that hypercalcemia could be bypassed by treatment with 25(OH)D_3. However, we and others [22,23] demonstrated a substantial reduction in 1α-hydroxylase activity in human PCa cell lines and cancer derived primary prostate epithelial cells compared to the cells derived from normal prostate or benign prostatic hyperplasia (BPH). Based on these findings we speculate that administration of 25(OH)D_3 to patients is unlikely to be effective as treatment of established PCa. However, the fact that normal cells exhibit high 1α-hydroxylase activity indicates that 25(OH)D_3 may be useful as a chemopreventive agent due to its local conversion to the active hormone within the normal prostate. The cause for the decreased expression of 1α-hydroxylase in cancer cells remains to be determined.

3.2. Differential sensitivity to calcitriol action and 24-hydroxylase (CYP24) expression

24-Hydroxylase or CYP24 is the enzyme that initiates calcitriol catabolism in target cells [12]. Calcitriol induces the expression of CYP24 in target cells. Therefore, calcitriol initiates its own inactivation. CYP24 is widely expressed in calcitriol target tissues including the prostate [16]. We and others have demonstrated that the anti-proliferative action of calcitriol varies among PCa cells and is inversely related to the level of CYP24 [16,18]. For example, the DU145 PCa cells, which are resistant to the growth inhibitory action of calcitriol, exhibit a high expression of CYP24. Treatment of the DU145 cells with liarozole, an inhibitor of P450 enzymes, sensitizes the cells to the anti-proliferative actions of calcitriol [24]. Similarly, Peehl et al. showed that the combined treatment with the general P450 inhibitor, ketoconazole and calcitriol or its analog EB 1089, increased their growth inhibitory actions [25]. These results strongly suggest that the combination of calcitriol with P450 inhibitors is a useful strategy to enhance the anti-cancer activity of calcitriol in PCa treatment.

4. Clinical studies of calcitriol effects in PCa

A pilot clinical study from our lab provides evidence that calcitriol effectively slows the rate of rise of serum prostatic specific antigen (PSA) in patients with early recurrent PCa after radical prostatectomy or radiation therapy [26]. However, the amount of calcitriol administered was limited by the development of hypercalciuria. Recent advances in the design of calcitriol analogs have resulted in potential drugs with increased potency and less tendency to cause hypercalcemia [2–11]. The beneficial effects of calcitriol have been observed only at supra-physiological concentrations (>1 nmol/L). These high concentrations might be more safely achieved without causing persistent hypercalcemia if calcitriol is administered intermittently [8,10]. Recent trials using intermittent high doses of calcitriol in combination with chemotherapy drugs have shown a beneficial effect in advanced PCa [27] with calcitriol potentiating the anti-tumor effects of many cytotoxic agents [28]. Trump et al. completed a phase II study of calcitriol and dexamethasone in AIPC. This study showed a significant slowing in PSA rise in 80% of the patients with the stabilization or decrease in PSA in 34% [10]. We believe that calcitriol or a new analog will prove to be a very useful adjunct for the therapy of both androgen-dependent PCa and AIPC. It is also possible that calcitriol therapy will prove to be useful in PCa chemoprevention.

5. Molecular mechanisms mediating anti-cancer response to calcitriol in PCa

The mechanisms governing the anti-proliferative actions of calcitriol are not fully known [2–11]. A number of important pathways have been shown to have a role in

calcitriol-mediated growth inhibition. A primary mechanism of calcitriol action is to induce cell cycle arrest in the G_1/G_0 phase. The growth arrest appears to be due to an increase in the expression of cyclin-dependent kinase inhibitors $p21^{Waf/Cip1}$ and $p27^{Kip1}$, a decrease in cyclin-dependent kinase 2 (Cdk2) activity, accompanied by a reduction in the nuclear fraction of this molecule and the hyperphosphorylation of the retinoblastoma protein (pRb). In addition, calcitriol induces apoptosis in some cells and down-regulates some anti-apoptotic genes, like bcl-2. Loss of the expression of cell cycle regulators has been associated with a more aggressive cancer phenotype, decreased prognosis and poorer survival. This suggests that calcitriol may be a suitable therapy to inhibit PCa progression [2–11]. Studies from our laboratory have implicated the increased expression of insulin-like growth factor binding protein-3 (IGFBP-3) in the growth inhibition induced by calcitriol which in turn increases $p21^{Waf/Cip1}$ expression [29]. Other mechanisms of calcitriol actions in PCa cells include the stimulation of differentiation, modulation of growth factor actions and the inhibition of invasion and metastasis [2–11]. Some in vivo studies have also demonstrated that the inhibition of angiogenesis contributes to the anti-tumor effects of calcitriol [2–11].

6. Novel calcitriol target genes

We performed cDNA-microarray analyses of normal and cancer-derived primary prostatic epithelial cells and LNCaP human PCa cells [30,31] to identify the molecular targets by which calcitriol mediates its effects on PCa cells. Table 1 presents some of the genes regulated by calcitriol in prostate cells. Our studies have recently shown that calcitriol regulates the expression of the genes involved in prostaglandin (PG) metabolism which has led us to hypothesize a new pathway for the anti-cancer activity of calcitriol, namely the regulation of PG metabolism [30,31].

6.1. Calcitriol effects on prostaglandin metabolism

Our microarray analyses showed that calcitriol up-regulates the expression of NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in LNCaP and normal primary prostate epithelial cells (see Fig. 1) [30,31]. Calcitriol also suppresses the expression of prostaglandin-endoperoxide synthase gene, also known as cyclooxygenase-2 (COX-2) in LNCaP cells (see Fig. 1). We have confirmed calcitriol regulation of these two genes by real time RT-PCR analysis. These data suggest that the regulation of PG metabolism could be an important molecular pathway of calcitriol action in prostate cells.

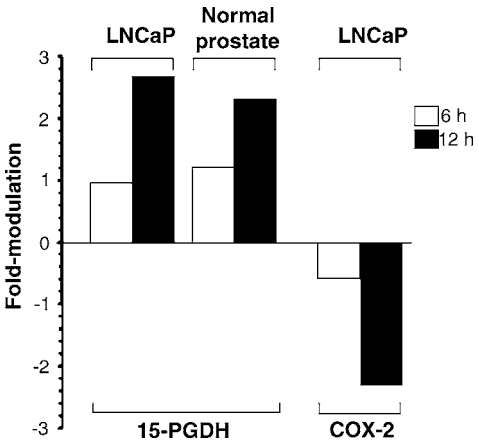

Fig. 1. Regulation of 15-PGDH and COX-2 by calcitriol in LNCaP cells and normal primary prostatic epithelial cells. LNCaP human prostate cancer cells or primary human prostatic epithelial cells derived from normal prostate were treated with calcitriol (50 nM) for 6 (□) or 24 h (■). $PolyA^+$ RNA was isolated and cDNAs were hybridized to a 24.192 element array. Data represent mean fold of increase or decrease of gene expression. (Adapted from Krishnan et al. [30] and Peehl et al. [31].)

6.1.1. COX-2

Prostaglandins play a role in the development and progression of PCa (for a review see Ref. [32]). Accumulating evidence implicates PGs as mediators of proliferation of PCa [33]. PG synthesis begins with the intracellular release of arachidonic acid (AA) from plasma membrane via the action of phospholipase A2. COX is the rate-limiting enzyme that catalyzes the conversion of AA to PGs [34]. The expression of COX-2 is rapidly induced by a variety of mitogens, cytokines, tumor promoters and growth factors, and therefore COX-2 is regarded as an immediate-early response gene [34]. Compelling evidence from genetic and clinical studies indicates that increased expression of COX-2 is one of the

Table 1
Genes modulated by calcitriol in prostate cells

Gene	Cell type	Fold change	
		6 h	24 h
Insulin-like growth factor binding protein-3	LNCaP	2.42	33.2
Vitamin D 24-hydroxylase	Normal prostate primary cells	79.1	82.5
Vitamin D 24-hydroxylase	PCa-derived epithelial cells	78.1	46.1
15-Hydroxyprostaglandin dehydrogenase	Normal prostate primary cells	1.2	2.3
15-Hydroxyprostaglandin dehydrogenase	LNCaP	0.95	2.66
Cyclooxygenase-2	LNCaP	−0.66	−2.13

Adapted from Krishnan et al. [30] and Peehl et al. [31].

key steps in carcinogenesis. Constitutive and high-levels of COX-2 activity have been detected in colon, lung, breast and prostate cancer [33,35]. It is known that high levels of PGE_2, one of the products of COX-2 activity, stimulate epithelial cell growth [33], promote cell survival and invasion [36]. Several studies have demonstrated COX-2 over-expression in prostate adenocarcinoma [32,37] suggesting a positive role for COX-2 in prostate tumorigenesis. COX-2 expression appears to be linked to cell survival. For example, COX-2 over-expression leads to the stabilization of survivin [38], a member of the inhibitor of apoptosis protein family that block caspase activation. The stabilization of survivin alters the balance between pro- and anti-apoptotic pathways leading to resistance to apoptosis. COX-2 expression has also been associated with angiogenesis and tumor metastasis [37] and the administration of a COX-2 inhibitor suppresses the growth of prostate tumor xenograft in mice by inhibiting angiogenesis [32]. We have found that calcitriol treatment decreases COX-2 gene expression in PCa cells (Fig. 1) [30]. Our data suggest that COX-2 suppression by calcitriol might contribute to the hormone's tumor inhibitory actions and anti-metastatic potential.

6.1.2. 15-PGDH

15-PGDH catalyzes the conversion of PGs into mostly inactive 15-keto derivatives [39]. A growing body of evidence has implicated 15-PGDH as a potential target for cancer therapy [40,41]. Various studies indicate that 15-PGDH acts as a putative tumor suppressor gene in lung cancer [41]. Backlund et al. [42] demonstrated a down-regulation of 15-PGDH expression in colorectal carcinoma. 15-PGDH has recently been described as an oncogene antagonist that functions as a tumor suppressor in colon cancer [43]. The study found that 15-PGDH, which physiologically antagonizes COX-2, was universally expressed in normal colon specimens but was routinely absent or severely reduced in cancer specimens. The study also showed that 15-PGDH expression was induced by transforming growth factor β (TGFβ) and that colon tumor cells exhibited mutations in TGFβ receptors or the genes involved in the SMAD signaling pathway explaining the decreased 15-PGDH levels in colon cancer. Most importantly, stable transfection of a 15-PGDH expression vector into cancer cells greatly reduced the ability of the cells to form tumors and/or slowed tumor growth in nude mice. The authors concluded that 15-PGDH suppressed the effects of the oncogene COX-2 and had an additional effect to inhibit angiogenesis in vivo [43]. Our studies show an up-regulation of 15-PGDH gene expression by calcitriol in normal and malignant prostate cells (Fig. 1) [30,31] and this regulation might mediate some of the anti-cancer effects of calcitriol. Interestingly, calcitriol has also been shown to induce the expression of 15-PGDH in human neonatal monocytes [44].

Based on our observations summarized in Table 1, we hypothesize that calcitriol treatment of PCa cells would reduce the levels of biologically active PGs due to its dual actions to reduce COX-2 expression and increase 15-PGDH expression. We further propose that the modulation of PG metabolism is an important molecular mechanism mediating the anti-proliferative and anti-metastatic activities of calcitriol.

7. Calcitriol and NSAIDs

Recent exciting findings show that non-steroidal anti-inflammatory drugs (NSAIDs) exert chemopreventive effects in several cancers [32,45]. NSAIDs have also been shown to suppress PCa development and progression [32,46]. The primary action of NSAIDs is to inhibit PG synthesis by directly inhibiting the enzymatic activity of COX-1 as well as COX-2. Many in vitro and in vivo studies have demonstrated that NSAIDs inhibit PCa growth and cause apoptosis of PCa cells [32,45]. However, many NSAIDs exhibit differences between their ability to inhibit COX-2 and to induce apoptosis suggesting that apoptosis induction may be independent of COX-2 inhibition [47]. The actions of NSAIDs on 15-PGDH are not clear. While many NSAIDs have been shown to inhibit the enzymatic activity of 15-PGDH [48], the non-selective NSAID indomethacin has been reported to enhance the expression and activity of 15-PGDH in thyroid carcinoma [49].

We hypothesize that the combination of calcitriol and NSAIDs would be additive/synergistic in their activity to inhibit PCa growth. The action of calcitriol at the genomic level to suppress COX-2 gene expression will decrease the levels of COX-2 protein and allow the use of lower concentrations of NSAIDs to inhibit COX-2 enzyme activity. The action of calcitriol to increase 15-PGDH expression would also complement NSAID action. The use of COX-2 selective NSAIDs has recently been shown to cause some serious cardiovascular side effects, such as increased risk of heart attacks, stroke, sudden death and blood clots [50]. The undesirable effect of calcitriol therapy is limited to hypercalcemia. We propose that a combination therapy of calcitriol with a NSAID would allow the use of lower concentrations of both drugs, thus reducing their individual side effects while increasing their anti-proliferative and pro-apoptotic activities.

8. Conclusions

Our research is directed at understanding the molecular mechanisms of the anti-proliferative activity of calcitriol in prostate cells with the goal of developing strategies to improve PCa treatment. Using cDNA microarrays we have recently found that calcitriol modulates the expression genes involved in PG metabolism. Calcitriol suppresses the expression of COX-2 gene, the enzyme that catalyzes PG synthesis and up-regulates the expression of 15-PGDH gene, the enzyme involved in PG inactivation. We hypothesize that

calcitriol treatment of PCa cells would reduce the levels of biologically active PGs due to its dual actions on COX-2 and 15-PGDH expression, and thereby decrease the PG-mediated proliferative stimulus. We further propose that the calcitriol effects would complement the action of NSAIDs, which are known inhibitors of both COX-1 and COX-2. The action of calcitriol at the genomic level to suppress COX-2 gene expression will decrease the levels of COX-2 protein and allow the use of lower the concentrations of NSAIDs needed to inhibit COX-2 enzyme activity. A combination of calcitriol with a NSAID would allow the use of lower concentrations of both drugs, thus reducing their individual side effects while increasing their anti-proliferative and pro-apoptotic activities. The combination approach is an attractive therapeutic strategy in the treatment of PCa and can be brought to clinical trials swiftly.

Note added in proof

Since this paper was originally written we have used real-time PCR and Western blot analyses to confirm that calcitriol inhibits PG actions in human PCa cell lines and primary prostatic epithelial cells. We found that calcitriol increased the expression of 15-PGDH, reduced the expression of COX-2, and reduced the expression of EP2 and FP PG receptors. These three calcitriol actions combine to decrease secreted PGE_2 and block cell growth stimulated by arachidonic acid and exogenous PGs. Moreover, calcitriol, in combination with various NSAIDs, produces synergistic inhibition of PCa cell growth at lower and safer drug concentrations. These data suggest that calcitriol and NSAIDs may be a useful combination for chemotherapy and/or chemoprevention of PCa. These new findings are now in press in Cancer Research 2005.

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Regulation of Prostaglandin Metabolism by Calcitriol Attenuates Growth Stimulation in Prostate Cancer Cells

Jacqueline Moreno,[1] Aruna V. Krishnan,[1] Srilatha Swami,[1] Larisa Nonn,[2] Donna M. Peehl,[2] and David Feldman[1]

Departments of [1]Medicine and [2]Urology, Stanford University School of Medicine, Stanford, California

Abstract

Calcitriol exhibits antiproliferative and prodifferentiation effects in prostate cancer. Our goal is to further define the mechanisms underlying these actions. We studied established human prostate cancer cell lines and primary prostatic epithelial cells and showed that calcitriol regulated the expression of genes involved in the metabolism of prostaglandins (PGs), known stimulators of prostate cell growth. Calcitriol significantly repressed the mRNA and protein expression of prostaglandin endoperoxide synthase/cyclooxygenase-2 (COX-2), the key PG synthesis enzyme. Calcitriol also up-regulated the expression of 15-hydroxyprostaglandin dehydrogenase, the enzyme initiating PG catabolism. This dual action was associated with decreased prostaglandin E_2 secretion into the conditioned media of prostate cancer cells exposed to calcitriol. Calcitriol also repressed the mRNA expression of the PG receptors EP2 and FP, providing a potential additional mechanism of suppression of the biological activity of PGs. Calcitriol treatment attenuated PG-mediated functional responses, including the stimulation of prostate cancer cell growth. The combination of calcitriol with nonsteroidal anti-inflammatory drugs (NSAIDs) synergistically acted to achieve significant prostate cancer cell growth inhibition at ~2 to 10 times lower concentrations of the drugs than when used alone. In conclusion, the regulation of PG metabolism and biological actions constitutes a novel pathway of calcitriol action that may contribute to its antiproliferative effects in prostate cells. We propose that a combination of calcitriol and nonselective NSAIDs might be a useful chemopreventive and/or therapeutic strategy in men with prostate cancer, as it would allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects. (Cancer Res 2005; 65(17): 7917-25)

Introduction

In the United States, prostate cancer remains the most common solid tumor malignancy in men, causing ~30,000 deaths in 2005 (1). Effective treatment options include surgery and radiation therapy. The main treatment strategy for advanced prostate cancer involves androgen deprivation therapy to which patients initially respond very well. However, most patients eventually fail this therapy and frequently develop metastatic disease. Current research on prostate cancer aims to identify new agents that would prevent and/or inhibit its progression.

1,25-Dihydroxyvitamin D_3 (calcitriol), the active form of vitamin D, is the major regulator of calcium and phosphate homeostasis in bone, kidney, and intestine (2). However, calcitriol has also been shown to exhibit antiproliferative and prodifferentiation effects in many normal and malignant cells including prostate cancer cells (3–10). There are multiple mechanisms underlying the antiproliferative effects of calcitriol, which vary between target cells (10). These include cell cycle arrest (9, 11) and the induction of apoptosis (12). Several genes that mediate these growth regulatory effects have been identified to be the molecular targets of calcitriol action, such as *p21, p27, bcl-2,* and insulin-like growth factor binding protein-3 (*IGFBP-3*; refs. 5–14). We recently did cDNA microarray analyses to more fully characterize the spectrum of genes regulated by calcitriol in prostate cells (15, 16). Among the newly identified genes regulated by calcitriol, we found two genes which play a key role in prostaglandin (PG) metabolism: the prostaglandin endoperoxide synthase-2 or cyclooxygenase (COX)-2 and the NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). PGs are synthesized from free arachidonic acid (17) by COXs. There are two well-characterized COX isoforms: COX-1, a constitutive form of the enzyme, and COX-2, an inducible form of the enzyme. PGs are implicated in the initiation and progression of many malignancies including prostate cancer (18–20). Tumor cells with elevated COX-2 levels are highly resistant to apoptosis, show increased angiogenic potential, and exert suppressive effects on host immunity (19, 20). Nonsteroidal anti-inflammatory drugs (NSAIDs), known inhibitors of both COX-1 and COX-2 enzymatic activity, are under intense investigation to prevent and/or treat malignancies (19, 21). 15-PGDH, which mediates the catalytic inactivation of PGs by converting them to the corresponding keto derivatives, has been found to be down-regulated in some cancers (22, 23) and has recently been regarded as a tumor suppressor gene (24).

In the current study, we investigated the regulation of COX-2 and 15-PGDH by calcitriol in the androgen-dependent LNCaP and androgen-independent PC-3 human prostate cancer cell lines as well as in primary prostatic epithelial cells derived from normal and cancerous human prostate tissue. Calcitriol reduced the expression of COX-2 and increased that of 15-PGDH. Calcitriol treatment of prostate cancer cells decreased the concentration of prostaglandin E_2 (PGE_2) secreted into the conditioned medium. In addition, calcitriol also decreased the expression of the mRNA of PG receptors EP2 and FP. Our data indicate that these mechanisms led to the attenuation of PG-mediated functional responses by calcitriol, including the suppression of PG stimulation of cell growth. Further, our study showed that the combination of calcitriol and NSAIDs exhibited synergistic growth inhibition, suggesting that the combination might be a useful therapeutic and/or chemopreventive strategy in prostate cancer.

Requests for reprints: David Feldman, Stanford University School of Medicine, Room S025, 300 Pasteur Drive, Stanford, CA 94305-5103. Phone: 650-725-2910; Fax: 650-725-7085; E-mail: feldman@cmgm.stanford.edu.

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Materials and Methods

Materials

PGE_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), arachidonic acid, NS-398, and SC-58125 were obtained from Cayman Chemical Co. (Ann Arbor, MI). Calcitriol was a gift from Leo Pharma A/S (Ballerup, Denmark). All stock solutions were made in 100% ethanol and stored at −20°C. Tissue culture media were obtained from Mediatech, Inc. (Herndon, VA). Tissue culture supplements and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Grand Island, NY).

Methods

Cell culture. LNCaP (ATCC no. CRL-1740) and PC-3 (ATCC no. CRL-1435) cells were grown in RPMI 1640 supplemented with 5% FBS, 100 IU/mL of penicillin, and 100 μg/mL streptomycin (Life Technologies). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. Primary cells were derived from radical prostatectomy specimens from men undergoing surgery for prostate cancer treatment. None of the patients had received prior therapy for prostate cancer. The normal cell strains (E-PZ-1 to -3) were derived from peripheral zone tissue with no histologic evidence of cancer in adjacent sections. The cancer cell strains used, E-CA-1 (Gleason grade 3/3), E-CA-2 (Gleason grade 4/5), and E-CA-3 (Gleason grade 4/3), were derived from adenocarcinoma specimens. Primary cell cultures were established from the prostate tissue samples and propagated in culture as we have previously described (25).

Cell proliferation assay. Prostate cancer cells were seeded at an initial density of 1.5×10^5 cells/well in six-well tissue culture plates and allowed to attach overnight in RPMI 1640 with 5% FBS. Cell cultures were shifted to medium containing 2% FBS and treated in triplicate over the next 6 days with either 0.1% ethanol vehicle or the indicated concentrations of drugs. Fresh media and the drugs were replenished every other day. At the end of the treatment, the cells were collected by gentle scraping and subjected to lysis in 0.2 N NaOH. Cell proliferation was assessed by the determination of DNA content (26).

RNA isolation and real-time reverse transcription-PCR. Total RNA was isolated from vehicle or drug-treated cells by the Chomczynski method using Trizol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA) as previously described (15). The yield and purity of isolated RNA were checked by UV spectrophotometry. Five micrograms of total RNA were used in reverse transcription reactions using the SuperScript III first strand synthesis kit (Invitrogen). Gene expression in vehicle or calcitriol-treated cells was determined by real-time PCR using the reverse transcription product and gene-specific primers. The reactions were carried out with the DyNamo SYBR Green qPCR kit (Finnzymes, Oy, M.J. Research, Reno, NV) in a 20 μL reaction volume containing gene-specific primers (10 pmol). All real-time PCR reactions were done in duplicate according to the following program: incubation at 72°C for 5 minutes, incubation at 95°C for 5 minutes, and 40 cycles of 94°C for 20 seconds, 58°C for 15 seconds, and 72°C for 20 seconds. PCR products were subjected to agarose gel electrophoresis to determine the purity and size of the amplified products (27). Real-time PCR was carried out using an Opticon 2 DNA engine (M.J. Research). Relative changes in mRNA expression levels were assessed by the $2^{-\Delta\Delta C(T)}$ method (28). Changes in mRNA expression of the different genes were normalized to that of TATA binding protein (*TBP*) gene or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The primers used were as follows: COX-2: 5′-GATACTCAGGCAGAGATGATCTACCC-3′ (sense), 5′-AGACCAGGCACCAGACCAAAGA-3′ (antisense); 15-PGDH: 5′-GACTCTGTTCATCCAGTGCG-3′ (sense), 5′-CCTTCACCTCCATTTTGCTTACTC-3′ (antisense); *c-fos*: 5′-GAATAAGATGGCTGCAGCCAAATGCCGCAA-3′ (sense), 5′-CAGTCAGATCAAGGGAAGCCACAGACATCT-3′ (antisense; ref. 29); EP2: 5′-GTGCTGACAAGGCACTTCATGT-3′ (sense), 5′-TGTTCCTCCAAAGGCCAAGTAC-3′ (antisense); FP: 5′-GCACATTGATGGGCAACTAGAA-3′ (sense), 5′-GCACCTATCATTGGCATGTAGCT-3′ (antisense); *TBP*: 5′-CACTCACAGACTCTCACAACTGC-3′ (sense), 5′-GTGGTTCGTGGCTCTCTTATC-3′ (antisense); *GAPDH*: 5′-GCCTCAAGATCATCAGCA-3′ (sense), 5′-GTTGCTGTAGCCAAATTC-3′ (antisense).

Measurement of prostaglandin E_2 secretion. Subconfluent LNCaP cells were treated with vehicle or calcitriol for 48 hours. Conditioned media were collected and secreted PGE_2 levels were quantitated using a PGE_2 monoclonal enzyme immunoassay kit (Cayman Chemical) according to the protocol of the manufacturer.

Western blot analysis. Cell lysates were prepared from vehicle or calcitriol-treated cells by lysis with a buffer containing 50 mmol/L Tris-HCl, 1 mmol/L EDTA, and 1.6 mmol/L CHAPS (Sigma-Aldrich, St. Louis, MO) supplemented with a protease inhibitor cocktail (Complet, Roche Diagnostics GmbH, Mannheim, Germany). Lysates were incubated at 4°C for 20 minutes and centrifuged at 10,000 × *g* for 1 minute to sediment particulate material. The protein concentration of the supernatant was measured by the Bradford method (30). Proteins were separated in either 10% NuPAGE gels in MOPS-SDS running buffer (Invitrogen) or 10% polyacrylamide Tris-Tricine (Sigma-Aldrich) gels depending on the size of the protein to be detected. After electrophoresis, proteins were transferred onto nitrocellulose membranes by electroblotting. The COX-2 monoclonal (1:1000 dilution) and 15-PGDH polyclonal antibodies (1:250 dilution) used in our study were purchased from Cayman Chemicals. β-Actin monoclonal antibody (dilution 1:500) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Membranes were incubated with the appropriate primary antibodies followed by incubations with a secondary antibody to immunoglobulin G conjugated to horseradish peroxidase (Cell Signaling Technology, Inc., Beverly, MA). Immunoreactive bands were visualized using the enhanced chemiluminescence Western blot detection system (Amersham, Piscataway, NJ) according to the instructions of the manufacturer. The blots were also probed for the expression of β-actin as a control. COX-2 protein was visualized as a ~70 kDa immunoreactive band. 15-PGDH protein was visualized as a ~29 kDa immunoreactive band. Immunoreactive bands were scanned by densitometry (HP Scanjet 7400C) and quantified using Bio-Rad software (Bio-Rad, Hercules, CA). COX-2 or 15-PGDH signals were normalized to β-actin levels in each sample.

Results

We previously showed by cDNA microarray analysis that calcitriol regulated the expression of two of the key genes involved in PG metabolism (i.e., COX-2 and 15-PGDH) in LNCaP human prostate cancer cells (15) and 15-PGDH in primary normal prostatic epithelial cells (16). In the present study, we extended these observations to include an evaluation of calcitriol effects on the expression of these two genes at both the mRNA and protein levels in LNCaP and PC-3 cells. In addition, we also examined the effects of calcitriol in primary prostatic epithelial cell strains derived from normal prostate as well as prostate adenocarcinoma specimens.

Down-regulation of cyclooxygenase-2 expression by calcitriol. Real-time reverse transcription-PCR (RT-PCR) analysis showed significant decreases in COX-2 mRNA levels in both androgen-dependent LNCaP (~70% inhibition) and androgen-independent PC-3 cells (~45% inhibition) due to calcitriol treatment (Fig. 1*A*). Although both LNCaP and PC-3 prostate cancer cells have been shown to express COX-2 protein (31), we found that PC-3 cells exhibited higher basal levels of COX-2 protein expression when compared with LNCaP cells (not shown). We therefore used PC-3 cells to assess the effect of calcitriol on COX-2 protein expression. Figure 1*B* shows that the addition of 10 nmol/L calcitriol to PC-3 cultures for 48 hours reduced COX-2 protein level to ~60% of control, with 100 nmol/L calcitriol having no further effect.

Up-regulation of 15-hydroxyprostaglandin dehydrogenase expression by calcitriol. We examined the effect of calcitriol on 15-PGDH mRNA levels in LNCaP and PC-3 cells. Our data show that 10 nmol/L calcitriol increased 15-PGDH mRNA expression by ~3.6-fold in LNCaP cells and by ~3-fold in PC-3 cells (Fig. 1*C*). We found that the basal protein expression of 15-PGDH varied between different cell lines with the LNCaP exhibiting appreciable levels of the 15-PGDH protein whereas barely detectable levels were seen in PC-3 cells. Therefore, we examined the effect of

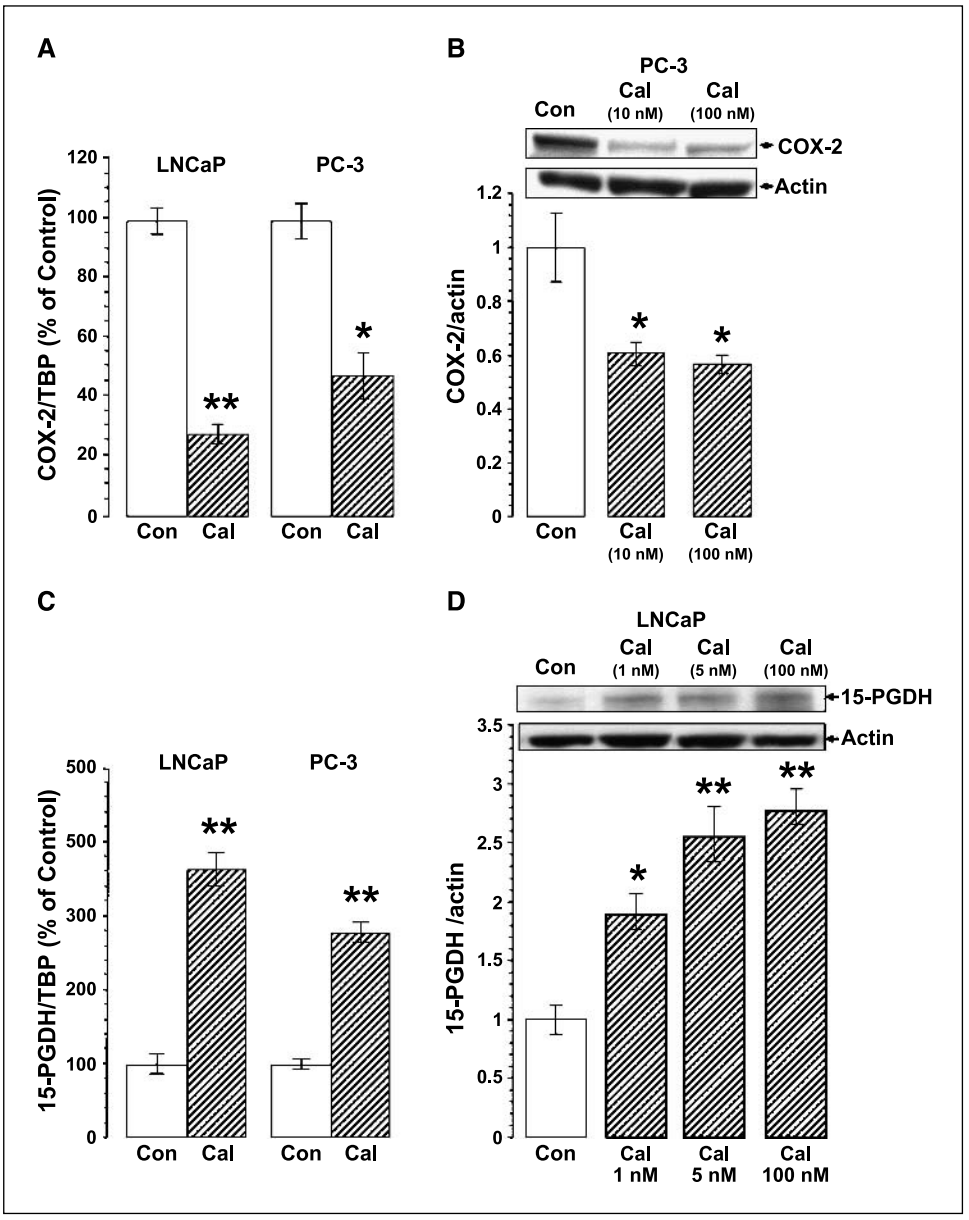

Figure 1. Calcitriol regulates COX-2 and 15-PGDH expression in prostate cancer cell lines. *A,* calcitriol decreases COX-2 mRNA levels. Subconfluent cultures of LNCaP and PC-3 cells were treated with 0.1% ethanol (*Con*) or 10 nmol/L calcitriol (*Cal*) for 24 hours and total RNA was extracted. COX-2 mRNA levels were determined by real-time RT-PCR as described in Materials and Methods and were normalized to *TBP* mRNA levels in the same samples. COX-2/TBP ratio shown as a percent of control set at 100%; *columns,* mean from five experiments; *bars,* SE. *B,* calcitriol decreases COX-2 protein levels. Subconfluent cultures of PC-3 cells were treated with 0.1% ethanol (*Con*) or 10 or 100 nmol/L calcitriol (*Cal*) for 48 hours. Fifty micrograms of total protein were subjected to Western blot analysis as described in Materials and Methods. Representative Western blot. The densitometric units of COX-2 immunoreactive bands were normalized to the densitometric units of the corresponding β-actin bands. Results expressed as the ratio of the control set at 1. *C,* calcitriol increases 15-PGDH mRNA levels. Cells were treated and processed as described in *A*. 15-PGDH/TBP ratio in calcitriol-treated cells given as a percent of control set at 100%; *columns,* mean from five experiments; *bars,* SE. *D,* calcitriol increases 15-PGDH protein levels. LNCaP cells were treated as in *B*. The densitometric units of 15-PGDH immunoreactive bands were normalized to the densitometric units of the corresponding β-actin bands. Results expressed as the ratio of the control set at 1; *columns,* mean of three experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, when compared with control.

calcitriol on 15-PGDH protein expression in LNCaP cells and found a dose-dependent increase in 15-PGDH protein levels in response to calcitriol treatment (Fig. 1*D*).

Calcitriol effects on cyclooxygenase-2 and 15-hydroxyprostaglandin dehydrogenase mRNA levels in primary prostatic epithelial cells. We extended our analysis to include calcitriol effects on primary cultures of prostatic epithelial cells derived from normal prostate as well as adenocarcinoma specimens removed at surgery. Real-time RT-PCR analysis showed considerable decreases (55-90%) in COX-2 mRNA levels in two of the three normal primary cell strains tested (E-PZ-1 and E-PZ-3) after 24 hours of calcitriol treatment (Fig. 2*A*). In all three cancer-derived primary cultures (E-CA-1 to -3) significant reductions (~48-60%) in COX-2 mRNA levels were seen at an earlier time point, after 6 hours of calcitriol treatment, and the down-regulatory effect was lost by 24 hours except in the case of E-CA-2 (Fig. 2*B*). Figure 2*C* and *D* shows the calcitriol-induced changes in 15-PGDH mRNA in primary prostatic cells. In the normal primary cells, calcitriol treatment caused appreciable increases in 15-PGDH mRNA in two of the three strains tested. The time course of this effect showed minor differences. In E-PZ-1 and E-PZ-2 cells significant increases (~2- to 18-fold) were achieved at the end of 6 and 24 hours, respectively (Fig. 2*C*). In two of three of the cancer-derived primary cultures (E-CA-2 and -3), significant increases (~2- to 5-fold) were seen at the end of 24 hours (Fig. 2*D*). In general, the magnitude of COX-2 mRNA down-regulation as well as 15-PGDH mRNA increase was more pronounced in the primary cells derived from normal prostatic tissue when compared with both the cancer-derived primary cells and the established prostate cancer cell lines.

Effect of calcitriol on prostaglandin levels. As a result of the dual action of calcitriol to down-regulate the expression of PG synthesizing COX-2 and increase the PG catabolizing 15-PGDH, we expected a reduction in PG production and secretion in prostate cancer cells treated with calcitriol. We measured the levels of PGE_2 in the conditioned media from LNCaP cells treated with various concentrations of calcitriol for 48 hours. Figure 3*A* shows that the

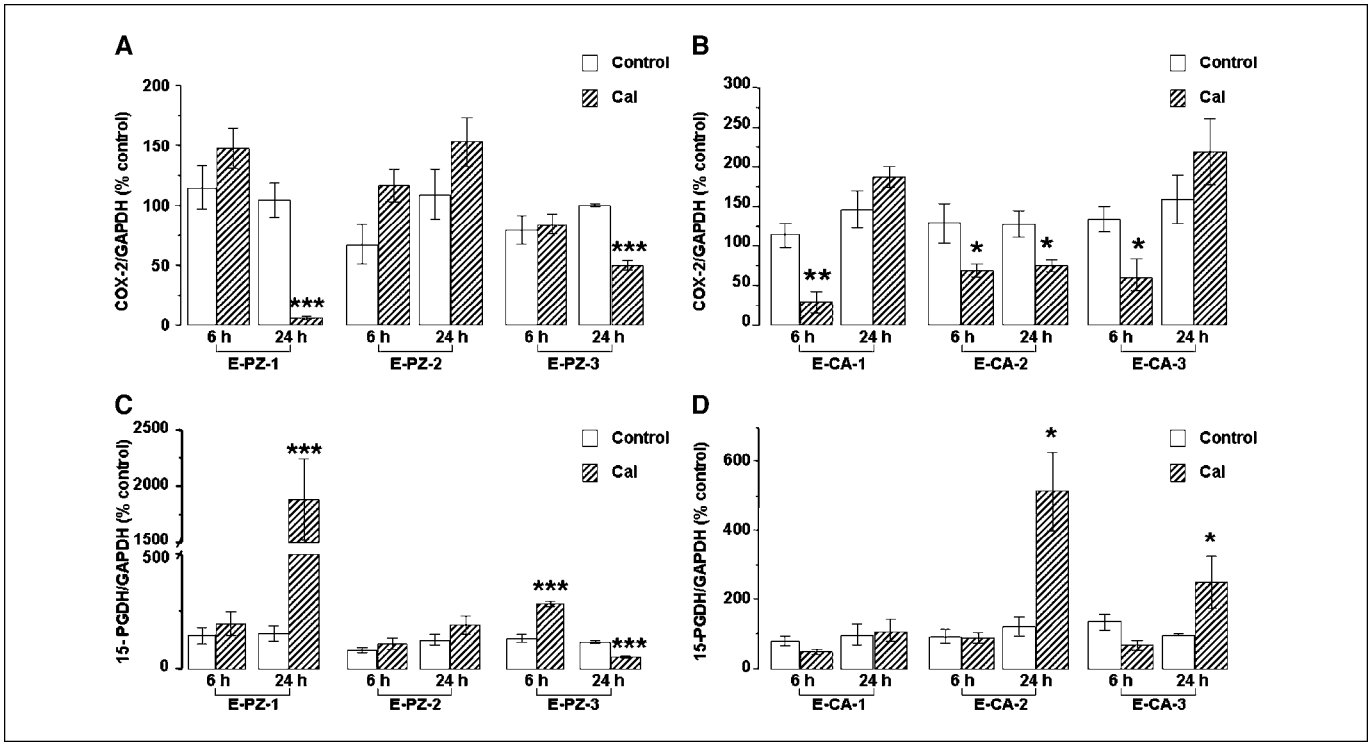

Figure 2. Calcitriol regulates the expression of COX-2 and 15-PGDH mRNA in primary prostatic epithelial cells. Primary cultures of prostatic epithelial cells derived from the peripheral zone of normal prostate tissue (E-PZ-1 to -3) or adenocarcinoma (E-CA-1 to -3) were treated with 0.1% ethanol (*Con*) or with 10 nmol/L calcitriol (*Cal*) for 6 or 24 hours. Total RNA was extracted and COX-2 and 15-PGDH mRNA levels were quantitated by real-time RT-PCR using gene-specific primers as described in Materials and Methods. COX-2 and 15-PGDH mRNA levels were normalized to *GAPDH* mRNA levels and are given as a percent of control set at 100%. *Columns,* mean from three experiments; *bars,* SE. Effect of calcitriol on COX-2 mRNA in three different normal primary epithelial cell strains (*A*) and in cancer-derived cell strains (*B*). Changes in 15-PGDH mRNA in normal cell strains (*C*) and in cancer-derived cell strains (*D*). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, when compared with control.

addition of calcitriol caused a significant reduction in PGE_2 secretion with the maximal decrease (~34%) seen with 100 nmol/L calcitriol.

Effects of calcitriol on prostaglandin receptor expression. Prostate cancer cells have been shown to express the PGE receptor subtypes EP2 and EP4 (29). We examined the effects of calcitriol on the expression of the PGE_2 receptor isoforms EP1, EP2, EP3, and EP4, and the $PGF_{2\alpha}$ receptor FP. LNCaP cells treated with 10 nmol/L calcitriol for 24 hours showed a significant (~45%) down-regulation of EP2 mRNA (Fig. 3*B*). We did not detect any changes in the levels of EP1, EP3, or EP4 mRNA following calcitriol treatment (not shown). FP mRNA levels were also down-regulated (~33% decrease) by calcitriol (Fig. 3*C*).

Inhibition of prostaglandin-mediated induction of *c-fos* mRNA by calcitriol. Because calcitriol modulated the levels of biologically active PGs as well as PG receptor expression, we examined its effect on a PG-mediated functional response, (i.e., the induction of the immediate-early gene *c-fos*; ref. 29). As serum is a potent inducer of *c-fos* expression (32), we conducted the experiment under serum-free conditions using PC-3 cells. Unlike LNCaP, PC-3 cells could be briefly maintained in serum-free media for calcitriol pretreatment and subsequent treatment with the PG precursor arachidonic acid. PC-3 cells were pretreated with vehicle or 10 nmol/L calcitriol for 48 hours followed by a brief (30 minutes) exposure to exogenous arachidonic acid (3 μmol/L) directly added to the culture medium. RNA was then isolated and the induction of *c-fos* mRNA was determined as an indicator of the biological activity of PGs endogenously synthesized from arachidonic acid. As shown in Fig. 3*D*, in vehicle pretreated cells arachidonic acid exposure resulted in a significant induction (~2.5-fold) of *c-fos* mRNA levels after 30 minutes. Calcitriol pretreatment completely abrogated the induction of *c-fos* mRNA due to arachidonic acid addition. Calcitriol pretreatment by itself caused a minor increase in *c-fos* mRNA levels when compared with vehicle pretreated cells, which was not statistically significant.

Effects of calcitriol on prostaglandin-mediated growth stimulation. We examined the effect of calcitriol on the stimulation of prostate cancer cell growth by exogenous PG addition as well as by endogenous PGs derived from the substrate arachidonic acid added to the culture medium. We treated LNCaP and PC-3 cells with arachidonic acid (3 μmol/L), PGE_2, or $PGF_{2\alpha}$ (10 μmol/L each) in the absence or presence of 10 nmol/L calcitriol. Our results revealed a moderate but significant growth stimulation by arachidonic acid and exogenous PGs in both LNCaP (Fig. 4*A*) and PC-3 cells (Fig. 4*B*). Calcitriol had a marked growth inhibitory action when given alone. In addition, calcitriol blocked the growth stimulation due to endogenous PGs derived from the added arachidonic acid as well as exogenous PG addition (Fig. 4*A* and *B*).

Synergistic inhibition of prostate cancer cell growth by calcitriol and nonsteroidal anti-inflammatory drugs. We next examined the combined effect of calcitriol and NSAIDs, which are potent inhibitors of COX enzyme activity. We tested a number of both COX-2–selective and nonselective NSAIDs including NS-398, SC-58125, flufenamic acid, sulindac sulfide, indomethacin, naproxen, and ibuprofen. Figure 5*A* to *D* illustrates the effect on prostate cancer cell growth of calcitriol alone

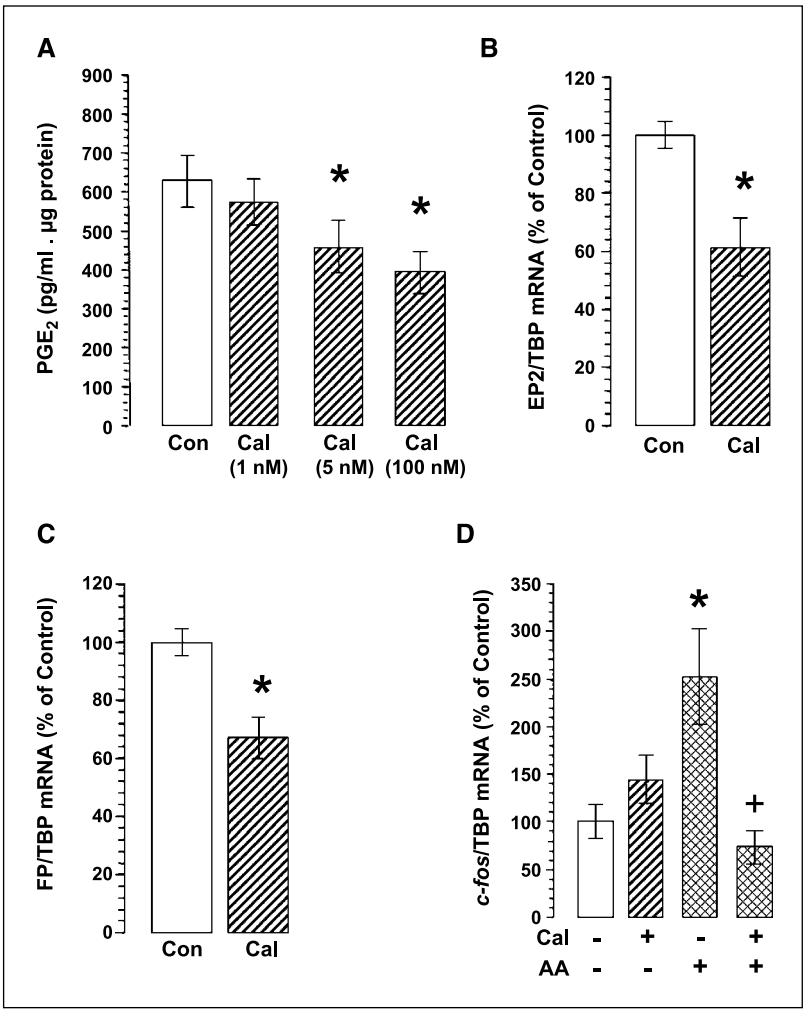

Figure 3. *A,* calcitriol decreases PGE_2 levels. Subconfluent cultures of LNCaP cells were treated with 0.1% ethanol (*Con*) or with the indicated concentrations of calcitriol (*Cal*) for 48 hours. Conditioned media from control and calcitriol-treated cultures were collected and PGE_2 levels were determined using an enzyme immunoassay kit (Materials and Methods). *Columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$. *B,* changes due to calcitriol treatment in EP2 mRNA. LNCaP cells were grown to subconfluence and treated with vehicle (0.1% ethanol; *Con*) or 10 nmol/L calcitriol (*Cal*) for 24 hours. Total RNA was extracted and analyzed for the mRNA expression of EP2 by real-time RT-PCR using gene-specific primers as described in Materials and Methods. EP2 mRNA levels were normalized to the *TBP* mRNA levels. Values given as a percent of control set at 100%; *columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$, when compared with control. *C,* changes due to calcitriol treatment in FP mRNA. LNCaP cells were treated and processed as in *B* for the mRNA expression of FP by real-time RT-PCR. FP mRNA levels were normalized to the *TBP* mRNA levels. Values are given as a percent of control set at 100%; *columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$, when compared with control. *D,* calcitriol inhibits PG-mediated induction of *c-fos* mRNA. Subconfluent cultures of PC-3 cells were transferred to serum-free RPMI 1640 containing 0.1% ethanol vehicle or 10 nmol/L calcitriol during 48 hours (pretreatment). Following the pretreatment, the cultures were exposed for 30 minutes to arachidonic acid (*AA*; 3 μmol/L) added to the culture medium. The cell cultures were then scraped, RNA was isolated, and *c-fos* mRNA levels were determined by real-time RT-PCR as described in Materials and Methods. *c-fos* mRNA levels were normalized to *TBP* mRNA levels and are given as a percent of control set at 100%. *Columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$, when compared with control; +, $P < 0.05$, when compared with arachidonic acid.

or in combination with the NSAIDs that exhibited the best growth inhibitory effect when used at a reduced dose. We show the effects of calcitriol alone and in combination with the COX-2–selective NSAIDs SC-58125 on the growth of LNCaP cells (Fig. 5*A*) and NS-398 on the growth of PC-3 cells (Fig. 5*B*). In LNCaP cells, calcitriol by itself had a modest effect (~20%) at 1 nmol/L but caused significant growth inhibition (~40%) at 10 nmol/L (Fig. 5*A*). The addition of the COX-2–specific inhibitor SC-58125 by itself had a modest effect on cell growth (~20% inhibition), which was not statistically significant, at the concentration tested (5 μmol/L). The combination of 1 nmol/L calcitriol with SC-58125, however, had a more pronounced inhibitory effect (~73% growth inhibition with the combination versus ~20% inhibition with the individual agents), indicating a synergistic interaction between these two drugs to inhibit cell growth. SC-58125 also enhanced the growth inhibition seen with the higher concentration of calcitriol (~80% inhibition with the combination versus ~40% inhibition with 10 nmol/L calcitriol alone). Similar synergistic growth inhibitory effects were evident in PC-3 cells treated with a combination of calcitriol and the COX-2–selective inhibitor NS-398 (Fig. 5*B*). NS-398, when used alone at 7.5 μmol/L, did not affect the growth of PC-3 cells. However, it enhanced the growth inhibition seen with both 1 and 10 nmol/L calcitriol (~60% inhibition with the combination versus ~20% inhibition with 1 nmol/L calcitriol alone, and ~75% inhibition with the combination versus ~40% inhibition with 10 nmol/L calcitriol alone).

The growth inhibitory effect of calcitriol was similarly enhanced when combined with nonselective NSAIDs that inhibit the enzymatic activity of both COX-1 and COX-2. The nonselective NSAID naproxen at 200 μmol/L did not inhibit the growth of LNCaP cells (Fig. 5*C*). However, it enhanced the growth inhibition seen with 1 and 10 nmol/L calcitriol (~65% inhibition with the combination versus ~48% inhibition with 10 nmol/L calcitriol alone). Similarly, in PC-3 cells (Fig. 5*D*), the nonselective NSAID ibuprofen at 150 μmol/L enhanced the growth inhibitory effect of calcitriol (~74% inhibition with the combination versus ~40% inhibition with 10 nmol/L calcitriol alone) whereas it did not affect cell growth when used alone at this concentration.

Based on extensive dose-response analysis (not shown), we calculated the interaction index (γ) using an isobolar method (33) for each drug combination. This analysis indicated a synergistic (superadditive) effect. The data suggested that ~2 to 10 times lower concentration of each drug is needed when used in combination to achieve the same degree of growth inhibition as achieved by the individual drugs.

Discussion

Calcitriol acts by multiple pathways to inhibit the proliferation of prostate cancer cells (5–14). Our study shows that the regulation

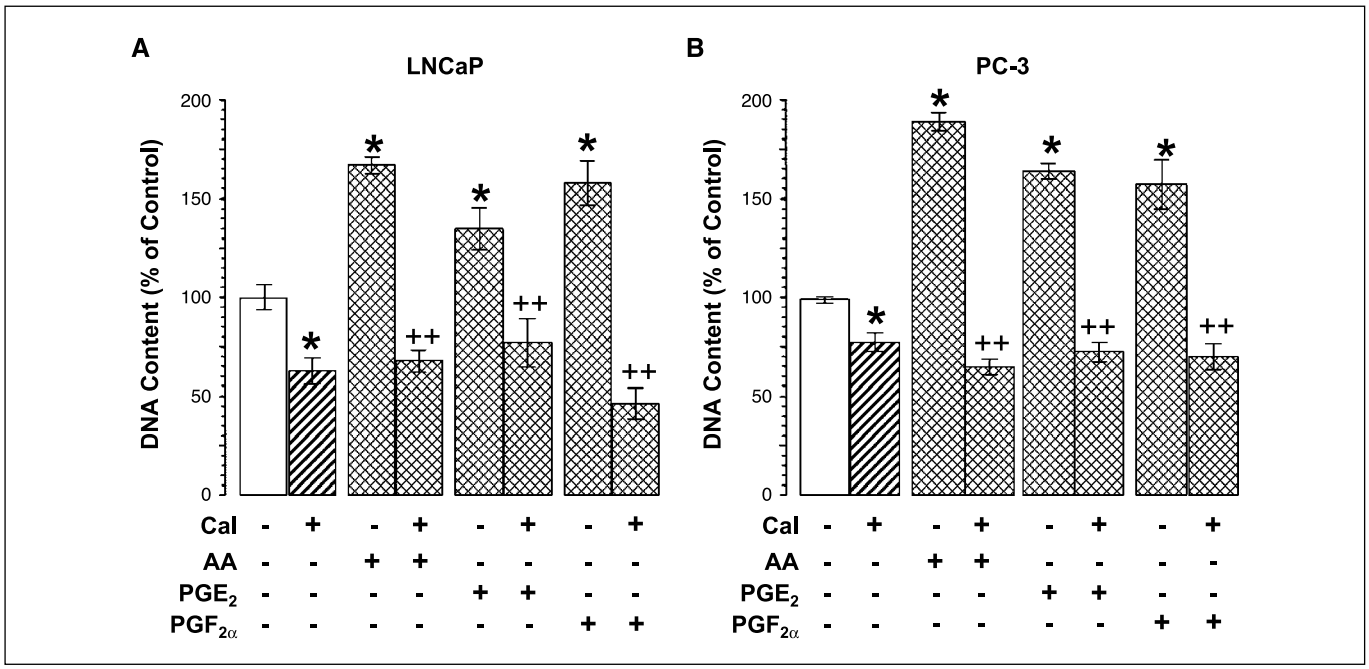

Figure 4. Calcitriol abrogates the growth stimulatory effects of arachidonic acid (*AA*) and exogenous PGs. LNCaP (*A*) and PC-3 (*B*) were treated with arachidonic acid (3 μmol/L), PGE_2 (10 μmol/L), or $PGF_{2\alpha}$ (10 μmol/L) individually or in combination with 10 nmol/L calcitriol (*Cal*) for 6 days. Cell growth was determined by measurement of DNA content as described in Materials and Methods. DNA contents are given as percentage of control value set at 100%, which was equivalent to 12.3 ± 1.2 μg/well for LNCaP cells and 19.3 ± 1.7 μg/well for PC-3 cells. *Columns,* mean from six experiments; *bars,* SE. *, $P < 0.05$, when compared with control; ++, $P < 0.01$, when compared with arachidonic acid, PGE_2, or $PGF_{2\alpha}$ alone.

of PG metabolism is a novel and additional pathway by which calcitriol may exert its antiproliferative actions in prostate cancer cells. We have shown that calcitriol regulates biologically active PG levels and PG actions by three mechanisms: (*a*) the suppression of COX-2 expression, (*b*) the up-regulation of 15-PGDH expression, and (*c*) the reduction of EP2 and FP PG receptor mRNA expression. We propose that these three effects act together to effectively inhibit the stimulation of prostate cancer cell proliferation by endogenously derived PGs as well as PGs added exogenously. Because PGs have been shown to promote prostate cell growth, inhibit apoptosis, and stimulate prostate cancer progression (18–20), we postulate that these effects of calcitriol to reduce PG actions significantly contribute to the anticancer effects of the hormone in prostate cancer.

The transformation of arachidonic acid into PGs and thromboxanes in mammalian cells is catalyzed by the enzyme COX, which has two well-characterized isoforms. COX-1 is constitutively expressed and is involved in housekeeping functions (17, 34). COX-2 is an immediate-early gene that is induced by a variety of growth promoting stimuli such as serum and growth factors, tumor promoters, cytokines, and proinflammatory agents (17, 34), and is regarded as an oncogene (24). COX-2 is overexpressed in various cancers including some, but not all, prostate cancers (18, 21). Inhibitors of COX-2 activity have been shown to suppress prostate cancer cell growth both *in vivo* and *in vitro* (31, 35, 36). Our results show the significant repression of COX-2 mRNA expression by calcitriol in prostate cancer cell lines as well as in primary prostatic epithelial cells and also a reduction in COX-2 protein levels in prostate cancer cell lines, suggesting that COX-2 is a calcitriol target gene.

PGE_2 and $PGF_{2\alpha}$ are rapidly catabolized *in vivo* into their biologically inactive 13,14-dihydro-15-keto metabolites by a two-step process carried out sequentially. The first step is initiated by the reversible oxidation of their 15(*S*)-hydroxyl group by the enzyme 15-PGDH (37). 15-PGDH is widely expressed in many mammalian tissues (38) and has been shown to be modulated by several hormones and factors (37–39), indicating the potential importance of the regulation of this enzyme. In LNCaP cells, 15-PGDH expression is up-regulated by androgens, interleukin-6, and the cyclic AMP inducer forskolin in a protein kinase A–dependent manner (40, 41). We now show that calcitriol is an important regulator of 15-PGDH expression in prostate cancer cells. The partial repression of COX-2 mRNA expression and the increase in 15-PGDH mRNA expression are also seen in primary prostatic epithelial cells derived from normal prostate, suggesting that these calcitriol effects are not restricted to malignant prostate cells. 15-PGDH expression has been shown to be decreased in many cancers (22, 23, 42). Calcitriol has also been shown to increase the expression of 15-PGDH in neonatal monocytes (43), where it exhibits prodifferentiation effects. 15-PGDH, which physiologically antagonizes COX-2, has recently been described as a putative oncogene antagonist that functions as a tumor suppressor in colon cancer by Yan et al. (24) who found that 15-PGDH was universally expressed in normal colon specimens but was routinely absent or severely reduced in cancer specimens. More importantly, stable transfection of a 15-PGDH expression vector into cancer cells greatly reduced the ability of the cells to form tumors and/or slowed tumor growth in nude mice. The authors concluded that 15-PGDH suppressed the effects of the oncogene COX-2 and exhibited an additional effect to inhibit angiogenesis *in vivo* (24). Our present study shows calcitriol-mediated suppression of the oncogene COX-2 and an increase in the expression of the putative tumor suppressor 15-PGDH in prostate cells, suggesting that calcitriol may play an important role in the chemoprevention of prostate cancer.

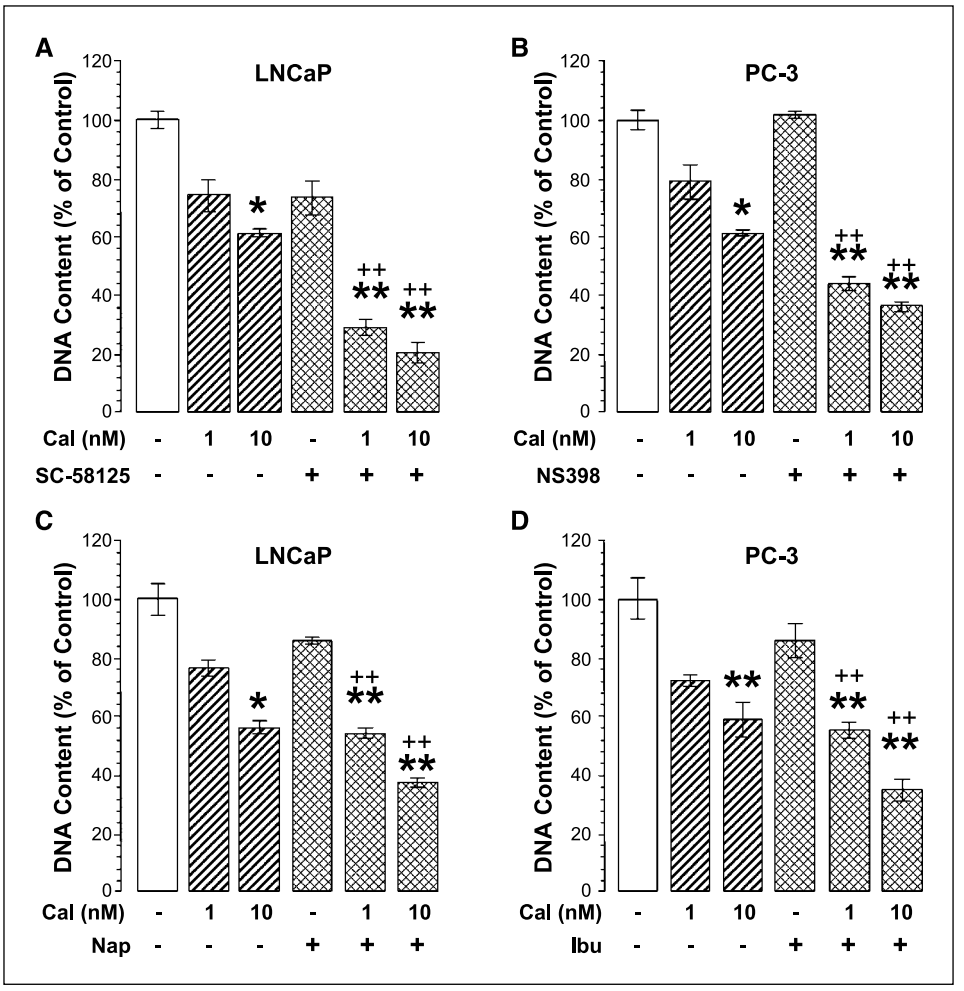

Figure 5. Synergistic inhibition of prostate cancer cell growth by calcitriol and NSAIDs. LNCaP or PC-3 cells were treated with 0.1% ethanol vehicle (*Con*) or 10 nmol/L calcitriol (*Cal*) in the presence and absence of the indicated NSAID. Cell growth was determined by measuring the DNA content as indicated in Materials and Methods. DNA contents are given as percentage of control value set at 100%. *A,* LNCaP cells treated with a combination of calcitriol (*Cal*) and COX-2–specific NSAID SC-58125 (5 μmol/L). 100% DNA content = 10.15 ± 1.22 μg/well. *B,* PC-3 treated with calcitriol (*Cal*) in the presence and absence of the COX-2–selective NSAID NS-398 (7.5 μmol/L). 100% DNA content = 17.42 ± 1.93 μg/well. *C,* LNCaP cells treated with calcitriol (*Cal*) in the presence and absence of the nonselective NSAID naproxen (*Nap*; 200 μmol/L). 100% DNA content = 9.22 ± 0.5 μg/well. *D,* PC-3 cells treated with calcitriol (*Cal*) in the presence and absence of the nonselective NSAID ibuprofen (*Ibu*; 150 μmol/L). 100% DNA content = 21.7 ± 0.9 μg/well. *Columns,* mean from six experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, when compared with control. ++, $P < 0.01$, when compared with 1 or 10 nmol/L Cal alone.

As a result of its dual action to modulate COX-2 and 15-PGDH expression, we expected calcitriol to reduce the levels of PGs in prostate cancer cells. This indeed was the case as shown by the decrease in PGE_2 levels in the conditioned media from LNCaP cells following calcitriol treatment. Calcitriol regulation of PGE_2 synthesis and secretion has been also reported in growth plate chondrocytes (44), in monocytes (43, 45), and in interleukin-1β–stimulated rheumatoid synovial fibroblasts (46). The effects of calcitriol on PG synthesis and signaling in these target cells seem to be related to the rapid nongenomic actions of calcitriol (47).

PGs exert their myriad effects through G-protein coupled membrane receptors which activate different signal transduction pathways (48). Prostate cancer cells have been shown to express the PGE receptor subtypes EP2 and EP4 (29). Interestingly, our study shows that calcitriol decreases the mRNA expression of the PGE_2 and $PGF_{2\alpha}$ receptor subtypes EP2 and FP, providing yet another mechanism for the suppression of the biological activity of PGs by calcitriol. In a recent study examining the changes in gene expression profile in the kidney of vitamin D receptor (VDR) knockout mice, Li et al. (49) report increases in the expression of EP3 and FP genes in $VDR^{-/-}$ kidneys, suggesting that calcitriol may also regulate the expression of PG receptors in kidney. Our study indicates that calcitriol not only decreases the concentration of PGs but may also inhibit the biological activity of these reduced PG levels by repressing of EP2 and FP receptor mRNA expression in prostate cancer cells.

Chen and Hughes-Fulford (29) have shown that arachidonic acid increases the expression of the immediate-early gene *c-fos* by undergoing a COX-2–mediated conversion to PGE_2, binding of PGE_2 to EP2/EP4 receptors, and subsequent activation of the protein kinase A pathway, which leads to the expression of growth-related genes. PGE_2 has also been to shown to up-regulate the gene expression of its own synthesizing enzyme COX-2 in prostate cancer cells, thereby completing a positive feedback loop (31, 50). We therefore examined the effect of calcitriol treatment on the induction of *c-fos* and cell growth by arachidonic acid in prostate cancer cells and found that calcitriol abolished *c-fos* induction and growth stimulation by arachidonic acid. Our interpretation of these observations is that they reflect both the effect of calcitriol to decrease endogenous synthesis of PGs due to COX-2 suppression and the ability of calcitriol to attenuate the biological activity of the PGs due to 15-PGDH up-regulation and EP and FP receptor down-regulation. The suppression by calcitriol of the growth stimulation by exogenous PG addition is probably due to its ability to enhance PG catabolism through the up-regulation of 15-PGDH expression as well as PG receptor down-regulation.

NSAIDs are known inhibitors of COX activity and have been shown to exhibit growth-suppressive effects in *in vivo* and *in vitro* models of prostate cancer (19, 35, 36, 50–52). The growth inhibitory and proapoptotic actions of NSAIDs are due to their ability to inhibit cyclooxygenase activity to a large degree, although in recent years mechanisms independent of COX-2 inhibition are also believed to

play a role (52). Our data show that the combination of calcitriol with COX-2–selective, as well as nonselective NSAIDs, acts synergistically to reduce the growth of prostate cancer cells. Our hypothesis is that the action of calcitriol at the genomic level to reduce COX-2 expression decreases the levels of COX-2 protein and allows the use of lower concentrations of NSAIDs to inhibit COX-2 enzyme activity, resulting in the enhanced growth inhibition seen with the combination. The potential use of NSAIDs as chemopreventive or therapeutic agents for a variety of malignancies, including prostate cancer, is being intensely investigated (20, 21, 51, 53). We propose that a combination of calcitriol and NSAID might be a useful therapeutic strategy in prostate cancer. The clinical use of NSAIDs has recently become controversial because of the cardiovascular complications associated with the use of high doses of COX-2–selective NSAIDs for prolonged periods of time (54, 55). In comparison with the COX-2–selective inhibitors, the use of a nonselective NSAID such as naproxen has been shown to be associated with decreased cardiovascular adverse effects (56). As shown by our study, an enhancement of growth inhibition is seen when calcitriol is combined with nonselective NSAIDs such as naproxen and ibuprofen. The clinical utility of the calcitriol combination with a nonselective NSAID is therefore worthy of evaluation, especially because the combination allows the use of lower concentrations of calcitriol and the NSAIDs, thereby improving the safety profile of the NSAIDs.

In conclusion, calcitriol acts by three separate mechanisms: decreasing COX-2 expression, increasing 15-PGDH expression, and reducing PG receptor mRNA levels. We believe that these actions contribute to suppress the proliferative stimulus provided by PGs in prostate cancer cells. The regulation of PG metabolism and biological actions constitutes an additional novel pathway of calcitriol action mediating its antiproliferative effects in prostate cells. We propose that a combination of calcitriol and a nonselective NSAID, such as naproxen, might be a useful therapeutic and/or chemopreventive strategy in prostate cancer, as it would achieve greater efficacy and allow the use of lower concentrations of both drugs, thereby reducing their toxic side effects.

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Review

Mechanisms of Vitamin D-mediated Growth Inhibition in Prostate Cancer Cells: Inhibition of the Prostaglandin Pathway

JACQUELINE MORENO[1], ARUNA V. KRISHNAN[1], DONNA M. PEEHL[2] and DAVID FELDMAN[1]

Departments of [1]Medicine and [2]Urology, Stanford University School of Medicine, Stanford, California 94305, U.S.A.

Abstract. *Calcitriol (1,25-dihydroxyvitamin D_3), the active form of vitamin D, promotes growth inhibition and differentiation in prostate cancer (PCa) cells. To unravel the molecular pathways of calcitriol actions, cDNA microarray analysis was used to identify novel calcitriol target genes including two that play key roles in the metabolism of prostaglandins (PGs), known stimulators of PCa growth and progression. Calcitriol significantly decreases the expression of the PG synthesizing cyclooxygenase-2 (COX-2) gene, while increasing that of PG inactivating 15-prostaglandin dehydrogenase (15-PGDH). Calcitriol also inhibits the expression of the PG receptors EP2 and FP. It reduces the levels of biologically active PGs and inhibits PG actions in PCa cells, thereby decreasing the proliferative stimulus of PGs. We postulate that the regulation of the PG pathway contributes to the growth inhibitory actions of calcitriol. We also propose that calcitriol can be combined with non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit COX enzyme activity, as a potential therapeutic strategy in PCa.*

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death in North American men (1). According to the American Cancer Society, more than 232,000 men will be diagnosed with PCa in 2005 and approximately 10% of these will die of the disease (1). Primary therapy for PCa involves the removal of the prostate by surgery or radiation therapy. Unfortunately, after initial treatment PCa often recurs. Androgens regulate normal prostate development and growth. Surgical or medical androgen deprivation has been used as the standard treatment for PCa which fails primary therapy (2). Although there is a good initial response to androgen ablation in most men, the tumors will progress to androgen independence resulting in death (3) since there is currently no adequate treatment for this advanced disease.

Correspondence to: Dr. David Feldman, Dept. of Medicine, Division of Endocrinology, Stanford University School of Medicine, Stanford, CA 94305, U.S.A. Tel: 650-725-2910, Fax: 650-725-7085, e-mail: feldman@cmgm.stanford.edu

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Our research was aimed at the development of new therapies for PCa. 1α, 25-Dihydroxyvitamin D_3 (calcitriol), the hormonally-active form of vitamin D, is a promising new therapeutic agent for PCa, which has been shown to exhibit growth inhibitory effects in cell culture and animal models of PCa as well as in clinical settings (4-15). Our goal was to understand the molecular mechanisms mediating the anticancer actions of calcitriol.

Calcitriol and PCa

Calcitriol is a steroid hormone, well known as a major regulator of calcium homeostasis and bone mineralization (16). However, data accumulated over the past 25 years have indicated that calcitriol and its analogs have potent antiproliferative and pro-differentiation actions in a number of malignancies including PCa (4-15, 17, 18). The anti-proliferative action of calcitriol has been documented in several PCa cell lines (6, 8, 17, 18), as well as in primary cultures of normal and cancer cells derived from surgical specimens of prostate obtained from PCa patients (19, 20). The inhibition of PCa cell growth is seen in both androgen-dependent and androgen-independent PCa cells (18, 21). Similarly, calcitriol and its analogs have been shown to inhibit the growth of PCa in animal models of PCa (8, 11, 12). A pilot clinical study by our group provided evidence that calcitriol effectively slowed the rate of serum PSA rise in PCa patients with early recurrent PCa (22). Recent clinical trials, using high doses of calcitriol in combination with chemotherapy, have shown great promise in prolonging survival and delaying the time to progression in men with androgen-independent PCa (10, 12). The only side-effect of calcitriol therapy appeared to be the development of hypercalcemia. Many pharmaceutical companies and academic centers are attempting to design calcitriol analogs that exhibit increased anticancer potency but

with a reduced tendency to cause hypercalcemia (23). We believe that calcitriol or a new analog will prove very useful as an adjunct for the therapy of both androgen-dependent and androgen-independent PCa.

Given the potential usefulness of calcitriol in treating and/or preventing PCa, understanding the molecular basis of calcitriol-mediated growth inhibition and the signaling pathways involved in its anticancer effects will more fully define its therapeutic potential, as well as allow the development of better therapeutic approaches to treat PCa and will provide an insight into how calcitriol acts and interacts with other agents in exerting its regulatory actions. This understanding would enable an improved rationale for when and how to implement calcitriol therapy and perhaps how to make calcitriol therapy more effective by combining with other drugs that exhibit synergistic molecular actions.

Mechanisms of Calcitriol Actions in PCa

Calcitriol exerts its actions by binding to its nuclear receptor, the vitamin D receptor (VDR). After hormone binding, VDR dimerizes with the retinoid X receptor (RXR). The VDR-RXR heterodimer binds to DNA sequences known as vitamin D response elements (VDREs) in the promoter regions of target genes. This calcitriol-VDR complex then recruits co-activator proteins that stimulate the transcriptional apparatus to induce the expression of the target gene. A number of important pathways have been shown to play key roles in calcitriol-mediated growth inhibition. One primary mechanism of calcitriol action is to induce cell cycle arrest in the G1/G0-phase by increasing the expression of genes like the cyclin-dependent protein kinase inhibitors p21 and p27 (8, 13, 24), and to induce apoptosis by down-regulating the expression of anti-apoptotic genes, such as bcl-2 (8, 11, 13). Calcitriol has also been shown to regulate growth factor action through modulation of the expression of genes such as insulin-like growth factor binding protein-3 (IGFBP-3) (8, 13) and transforming growth factor β (TGF-β) (11, 13). In addition, calcitriol also exerts inhibitory effects on tumor cell migration and metastasis, as well as tumor angiogenesis (8, 10, 11, 13).

Novel Targets of Calcitriol in PCa

Using cDNA microarray analysis to study the regulation of gene expression by calcitriol, we have recently identified 28 genes regulated by calcitriol in LNCaP human PCa cells (25). Among the up-regulated genes is one that encodes NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). The expression of 15-PGDH is also induced by calcitriol in primary cultures of normal prostatic epithelial cells (26). We found the down-regulation of various genes on our microarrays, including the prostaglandin-endoperoxide synthase-2, or cyclooxygenase-2 (COX-2) gene (25). COX-2 is the rate-limiting enzyme involved in prostaglandin (PG) synthesis, while 15-PGDH is the primary enzyme responsible for PG catabolism.

COX-2 and PCa

PGs are long-chain oxygenated polyunsaturated fatty acids derived from arachidonic acid (AA). COX or cyclooxygenases are responsible for the synthesis of the PG precursor PGH_2 from AA (27). PGH_2 is then converted to the various PGs by specific synthases. PGs have been shown to stimulate the proliferation of many cancers including PCa (28). Many, yet not all, studies have concluded that the expression of COX-2 is elevated in PCa when compared with normal prostate (29-32). *In vitro* studies using androgen-dependent and androgen-independent PCa cell lines showed that both expressed detectable amounts of COX-2 and secreted PGE_2 (29, 33). It has been proposed that COX-2 induces tumorigenesis by various mechanisms including: (i) induction of cell proliferation (34); (ii) decreased apoptosis (35); (iii) increased angiogenesis (36); (iv) increased tumor invasiveness (37); and (v) decreased immune surveillance (33). Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX enzymatic activity and therefore PG synthesis, have been shown to decrease PCa growth in *in vitro* PCa cell cultures and *in vivo* in animals bearing PCa tumor xenografts (28, 35, 38). Existing evidence suggests that PGE_2 has a specific role in the maintenance of human cancer cell growth and that the activation of COX-2 expression depends primarily upon newly synthesized PGE_2 through a positive feedback mechanism (39). Taken together, these data indicate that COX-2 and/or their prostaglandin products play a role in the malignant transformation of the prostate.

15-PGDH and Cancer

15-PGDH is the key enzyme initiating the catabolism of biologically-active PGs converting them into inactive keto-derivatives (40), thereby acting as a functional antagonist to COX-2. Three pieces of evidence indicate that the concomitant overexpression of COX-2 and underexpression of 15-PGDH have a role in tumor progression. First, microarray data analysis indicated a down-regulation of 15-PGDH in colon (41) and lung (42) cancers when compared to normal tissues. Second, when colon epithelial cells were chronically treated with the tumor suppressor TGF-β, 15-PGDH gene expression was induced (41). Thirdly, 15-PGDH by itself seemed to have tumor suppressor effects (42). When lung cancer cells transiently overexpressing 15-PGDH were injected into athymic nude mice, there was a substantial decrease in tumor induction and growth when compared to mice implanted with wild-type cancer cells (42).

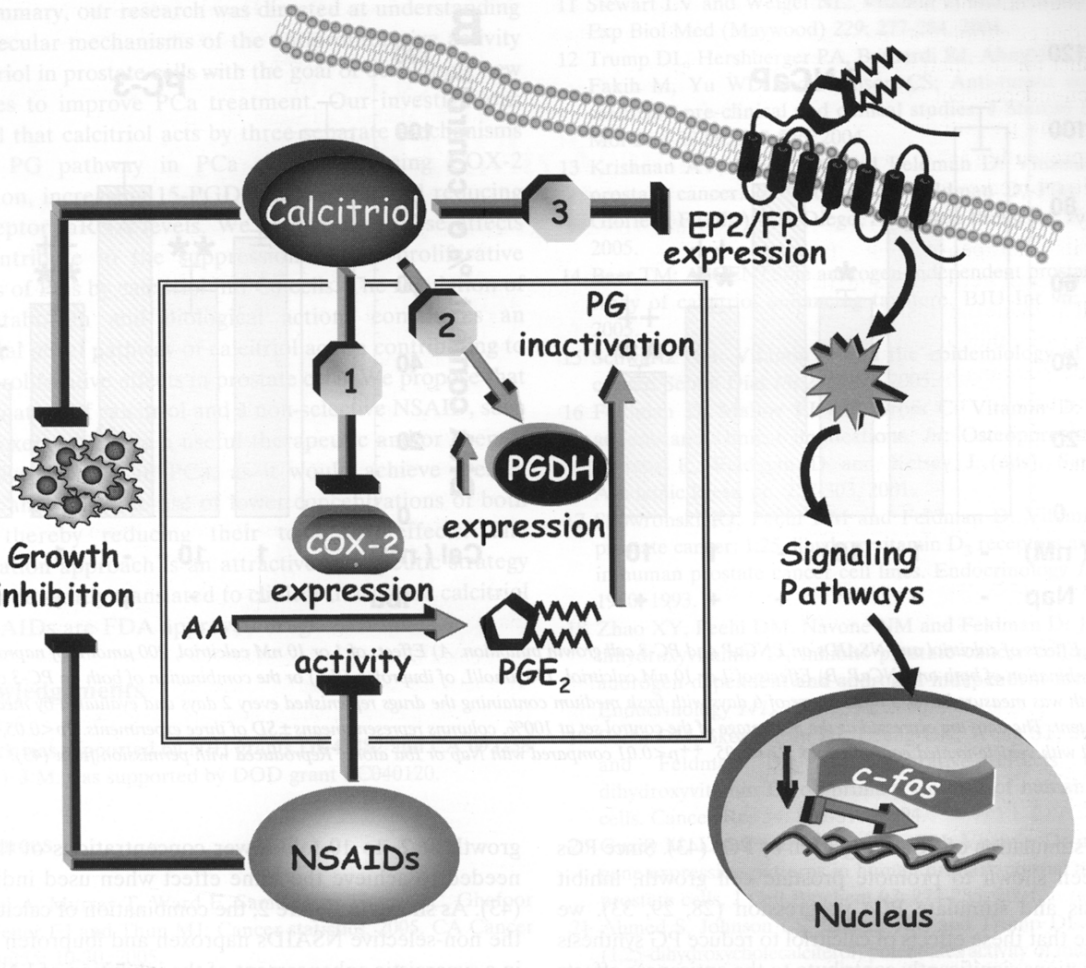
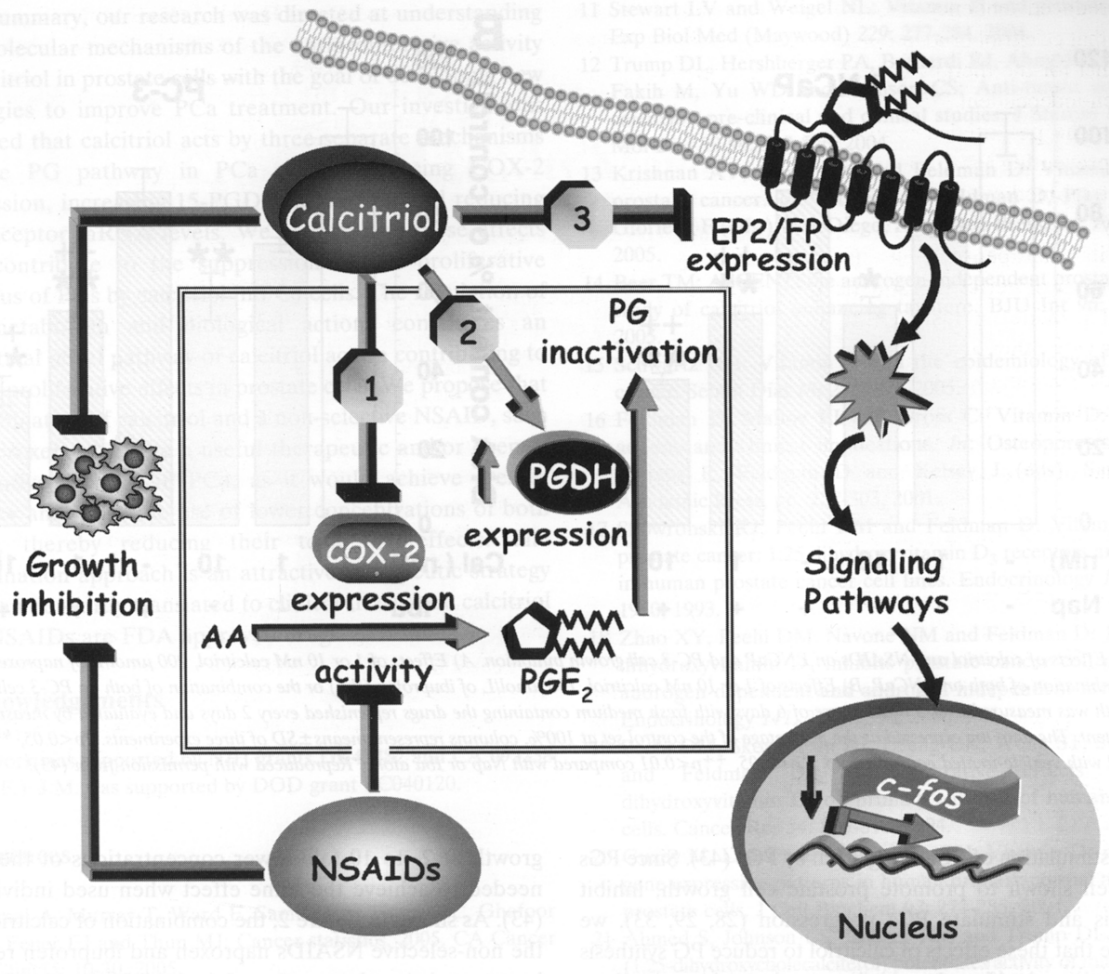


Figure 1. *Calcitriol inhibits prostaglandin actions in prostate cancer cells by three mechanisms: i) decreasing the expression of the prostaglandin synthesizing enzyme cyclooxygenase-2 (COX-2); ii) stimulating the expression of the prostaglandin catabolizing enzyme 15-prostaglandin dehydrogenase (PGDH); and iii) inhibiting EP2 and FP prostaglandin receptor expression. These three actions combined may be involved in blocking PG-mediated actions such as c-fos induction and cell growth. Furthermore, the combination of calcitriol and various non-steroidal anti-inflammatory drugs (NSAIDs) produced synergistic inhibition of prostate cancer cell growth.*

Calcitriol Actions on the Prostaglandin Pathway in PCa cells

Our microarray data indicated that calcitriol increased the expression of 15-PGDH and significantly decreased the expression of COX-2 in LNCaP human PCa cells (25). Based on these initial results, we further investigated the effect of calcitriol on PG metabolism and PG actions in several established PCa cell lines, as well as primary cultures of prostatic epithelial cells derived from normal prostate and adenocarcinoma specimens removed at surgery. Significant increases were found in 15-PGDH expression and down-regulation of COX-2 expression in both the PCa cell lines and primary prostatic cells (43). This dual action was associated with decreased PGE_2 secretion into the conditioned media of PCa cells exposed to calcitriol (43).

PGs exert their biological effects through G-protein coupled membrane receptors which activate different signal transduction pathways. Interestingly, our study showed that calcitriol decreased the mRNA expression of the PGE_2 and $PGF_{2\alpha}$ receptor sub-types EP2 and FP, providing yet a third mechanism for the suppression of the biological activity of PGs by calcitriol (43). Thus, as illustrated in Figure 1, calcitriol exerts multiple actions on the PG pathway: the suppression of PG synthesis (due to COX-2 down-regulation), increase of PG catabolism (due to 15-PGDH up-regulation) and the inhibition of PG actions (due to PG receptor down-regulation). We hypothesize that, as a result of these regulatory actions, calcitriol attenuated PG-mediated functional responses in PCa cells such as the induction of the immediate-early gene *c-fos*



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In summary, our research was directed at understanding the molecular mechanisms of the antiproliferative activity of calcitriol in prostate cells with the goal of developing new strategies to improve PCa treatment. Our investigations revealed that calcitriol acts by three separate mechanisms on the PG pathway in PCa cells: decreasing COX-2 expression, increasing 15-PGDH expression and reducing PG receptor mRNA levels. We believe that these effects may contribute to the suppression of the proliferative stimulus of PGs by calcitriol in PCa cells. The regulation of PG metabolism and biological actions constitutes an additional novel pathway of calcitriol action contributing to its antiproliferative effects in prostate cells. We propose that a combination of calcitriol and a non-selective NSAID, such as naproxen, might be a useful therapeutic and/or chemopreventive strategy in PCa, as it would achieve greater efficacy and allow the use of lower concentrations of both drugs, thereby reducing their toxic side-effects. The combination approach is an attractive therapeutic strategy that can be swiftly translated to clinical trials since calcitriol and NSAIDs are FDA approved drugs.

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PATHWAYS MEDIATING THE GROWTH INHIBITORY ACTIONS OF VITAMIN D IN PROSTATE CANCER

David Feldman, Jacqueline Moreno and Aruna Krishnan,

Department of Medicine/Endocrinology, Stanford University School of Medicine, Stanford CA 94305-5103

Vitamin D is emerging as an important hormone that affects the development and progression of many malignancies including prostate cancer (PCa). 1,25-dihydroxyvitamin D3 (calcitriol), the active form of vitamin D, inhibits the growth and stimulates the differentiation of PCa cells. We have studied established human PCa cell lines as well as primary cultures of normal or cancer-derived prostatic epithelial cells to elucidate the molecular pathways of calcitriol actions. These pathways are varied and appear to be cell-specific. We have used cDNA microarray analysis to elucidate additional genes regulated by calcitriol in order to identify novel therapeutic targets for the treatment of PCa. Several potentially useful target genes have emerged from these studies. In this talk I will highlight two new target genes, both involved in prostaglandin (PG) metabolism. Accumulating evidence has implicated PGs in stimulating the development of many types of cancer including PCa. PGs have been associated with the progression of PCa, tumor invasiveness and tumor grade. Prostatic PGs are formed by the action of the cyclooxygenase enzyme COX-2. The first step in PG inactivation is mediated by 15-hydroxyprostaglandin dehydrogenase (PGDH). We found that calcitriol down-regulates the expression of COX-2 and up-regulates PGDH. Currently there is much interest in the use of COX-2 inhibitors to prevent and/or treat PCa, due to their ability to inhibit growth and induce apoptosis. Moreover, PGDH has recently been proposed as a tumor suppressor. The actions of calcitriol to induce PGDH and inhibit COX-2, constitute a pathway to reduce and/or remove bioactive PGs thereby diminishing PCa proliferation. Treatment of LNCaP cells with a combination of calcitriol and COX-2 inhibitors resulted in synergistic growth inhibition. In combination, calcitriol and COX-2 inhibitors allowed the use of reduced doses of both drugs that still resulted in enhanced antiproliferative activity. These findings suggest that therapy combining calcitriol and COX-2 inhibitors will increase the efficacy of both drugs while decreasing their side-effects. We propose that this combination of already approved drugs can be brought to clinical trial swiftly, particularly in patients with early recurrent PCa that demonstrate rising PSA after primary therapy. In conclusion, our research is directed at understanding the mechanisms of vitamin D action in prostate cells with the goal of developing treatment strategies to improve PCa therapy. The ability of calcitriol to inhibit PG synthesis and stimulate PG inactivation appears to be an additional pathway by which calcitriol can enhance PCa therapy.

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ABSTRACTS OF THE SECOND INTERNATIONAL SYMPOSIUM ON VITAMIN D ANALOGS IN CANCER PREVENTION AND THERAPY

7-8 May 2005, Lübeck, Germany

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amount of UV radiation from natural sunlight and the exposure of only small parts of the body, *e.g.* hands, forearms and/or face, are necessary. The aim of this study was to evaluate the correlation between Vitamin D-weighted UV dosage (H_{vd}) *versus* the increase of circulating $25(OH)D_3$ and $1,25(OH)_2D_3$. *Patients and Methods*: Twenty-two dialysis patients were partial body (frontal part of the legs, approx. 15% of body surface) irradiated over a period of 14 weeks using an artificial UV-source (UVB 3.5%); blood samples were taken every two weeks. *Results*: The peak value of $25(OH)D_3$ was found after 8 weeks (increase Δ + 13µg/l = +33%, median) and the peak of $1,25(OH)_2D_3$ followed 6 weeks later (increase Δ + 9ng/l = +90%). Therefore, the following algorithm can be calculated: $25(OH)_2D_3 = (H_{vd}^2 \times 10^5)+25$, as a nonlinear correlation ($r^2=0.992$); following a linear correlation (r=0.32) between $25(OH)D_3$ and $1,25\ (OH)_2D_3$. *Conclusion*: A sufficient pool of circulating $25(OH)D_3$ is necessary for conversion to $1,25(OH)_2D_3$. In renal patients, the threshold level of $25(OH)D_3$ seems to be ≥35 µg/l.

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EFFECT OF UVB RADIATION EMITTED FROM THE NARROWBAND TL-01 LAMP (311 NM) ON CALCITRIOL SYNTHESIS IN ORGANOTYPIC CULTURES OF KERATINOCYTES

Bodo Lehmann and Michael Meurer

Department of Dermatology, Carl Gustav Carus Medical School, Dresden University of Technology, Dresden, Germany

The skin is the only tissue known in which the complete UVB-induced pathway from 7-dehydrocholesterol (7-DHC) to hormonally-active calcitriol (1α,25-dihydroxyvitamin D_3) occurs under physiological conditions. It is well known that both calcitriol and UVB radiation exert potent antipsoriatic effects. We speculate that the therapeutic effect of UVB radiation can be attributed to UVB-triggered cutaneous synthesis of calcitriol, for which the optimum wavelength was 300 ± 3 nm *in vitro* and *in vivo*. On the other hand, the narrowband Philips TL-01 lamp, which is commonly used as a UVB source for the treatment of psoriasis, has a maximum spectral irradiance at around 311 nm. The aim of this study was to investigate the calcitriol-inducing potential of the TL-01 lamp in organotypic cultures of keratinocytes supplemented with 25 µM 7-DHC at different radiant exposures (125-1000 mJ/cm^2). We found that the maximum calcitriol-generating capacity of the TL-01 lamp at 500 mJ/cm^2 (corresponding to 2.1 SED [Standard Erythema Dose]) and 16 hours after irradiation still amounted to approximately 45% of that of monochromatic radiation at 300 nm and 30 mJ/cm^2. We conclude that irradiation with the narrowband TL-01 lamp in a therapeutic dose range can affect calcitriol synthesis in epidermal keratinocytes. Thus, the antipsoriatic effect observed after TL-01 lamp exposures may be, at least partially, explained by the known action of newly-synthesized calcitriol on epidermal cell proliferation and differentiation.

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PATHWAYS MEDIATING THE GROWTH INHIBITORY ACTIONS OF VITAMIN D IN PROSTATE CANCER

Jacqueline Moreno, Aruna Krishnan and David Feldman

Department of Medicine/Endocrinology, Stanford University School of Medicine, Stanford, CA 94305-5103, U.S.A.

Vitamin D is an important hormone that affects the incidence and progression of many malignancies including prostate cancer (PCa). 1,25-dihydroxyvitamin D (calcitriol), the active form of vitamin D, inhibits the growth and stimulates the differentiation of PCa cells. We studied established human PCa cell lines as well as primary cultures of normal or cancer-derived prostatic epithelial cells to elucidate the molecular pathways of the action of calcitriol. These pathways are varied and some appear to be cell-specific. We used cDNA microarray analysis to ascertain additional genes regulated by calcitriol, in order to identify novel therapeutic targets for the treatment of PCa. Several potentially useful target genes have emerged from these studies including two new target genes, both involved in prostaglandin (PG) metabolism.

Accumulating evidence has implicated PGs in stimulating the development of many types of cancer including PCa. PGs have been associated with the progression of PCa, tumor invasiveness and tumor grade. Prostatic PGs are formed by the action of the cyclooxygenase enzyme COX-2. The first step in PG inactivation is mediated by 15-hydroxyprostaglandin dehydrogenase (PGDH). We found that calcitriol down-regulates the expression of COX-2 and up-regulates PGDH. There is much current interest in the use of second-generation COX-2 inhibitors or non-selective nonsteroidal anti-inflammatory drugs (NSAIDs), to prevent and/or treat PCa, due to their ability to inhibit growth and induce apoptosis. Moreover, PGDH has recently been proposed as a tumor suppressor. The actions of calcitriol to induce PGDH and inhibit COX-2 constitute a pathway to reduce and/or remove active PGs, thereby diminishing PCa proliferation. Combination therapy of LNCaP cells with calcitriol and NSAIDs revealed synergistic growth inhibition. In combination, calcitriol and NSAIDs allowed the use of

reduced doses of both drugs that still resulted in enhanced antiproliferative activity. These findings suggest that therapy combining calcitriol and NSAIDs will increase efficacy while decreasing side-effects. We propose that this combination of already approved drugs can be brought to clinical trial swiftly, particularly in patients with early recurrent PCa that demonstrate rising PSA after primary therapy. In conclusion, our research is directed at understanding the mechanisms of vitamin D action in prostate cells with the goal of developing treatment strategies to improve PCa therapy. The ability of calcitriol to inhibit PG synthesis and stimulate PG destruction appears to be an additional pathway by which calcitriol can enhance PCa therapy.

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BIOLOGICAL EFFECTS OF 1α,25-DIHYDROXYVITAMIN D_3 ON HUMAN KERATINOCYTES AFTER IONIZING RADIATION

Kerstin Müller, Michaela Schinn, Jörg Reichrath and Viktor Meincke

Bundeswehr Institute of Radiobiology, Munich; Department of Dermatology, The Saarland University Hospital, Homburg, Germany

Exposure of human skin to ionizing radiation results in various early and late effects such as an inflammatory reaction, keratosis, fibrosis, radiation vasculitis and cancer. 1α,25-Dihydroxyvitamin D_3, the biologically active metabolite of vitamin D, has been shown to exert pleiotropic effects in the skin. We evaluated whether the radiation reaction of human keratinocytes (HaCaT cells) can be modulated by 1α,25-dihydroxyvitamin D_3. The cell growth of keratinocytes after ionizing radiation was significantly increased in the presence of 1α,25-dihydroxyvitamin D_3 as compared to the untreated control. Moreover, 1α,25-dihydroxyvitamin D_3 also exerted a positive influence on the cell survival of irradiated keratinocytes, as shown by clonogenic assay. As the cutaneous radiation reaction is determined by various inflammatory parameters, including adhesive interactions mediated by cellular adhesion molecules, we analyzed the cell surface expression of intercellular adhesion molecule-1 (ICAM-1) and β1-integrin in keratinocytes and the effect of 1α,25-dihydroxyvitamin D_3 using flow cytometry and immuno- histochemistry. The results revealed that ionizing radiation causes an up-regulation of both ICAM-1 and β1-integrin in keratinocytes, which was inhibited by pretreatment of the cells with 1α,25-dihydroxyvitamin D_3. Taken together, our data suggest that 1α,25-dihydroxyvitamin D_3 might be a promising agent to modify the radiation reaction, offering new options in radiotherapy and oncology.

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SNAIL REPRESSES VITAMIN D RECEPTOR EXPRESSION AND BLOCKS THE EFFECTS OF 1α,25-DIHYDROXYVITAMIN D_3 ON HUMAN COLON CANCER CELLS *IN VITRO* AND *IN VIVO*

A. Muñoz, H.G. Pálmer, M.J. Larriba and P. Ordóñez-Morán

Instituto de Investigaciones Biomédicas, Madrid, Spain

We have previously reported that 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) and several non-hypercalcemic analogs (EB1089, MC903 and KH1060) inhibit proliferation and promote differentiation of human SW480-ADH colon cancer cells. They induce the expression of E-cadherin and the translocation of β-catenin from the nucleus to the plasma membrane. The Wnt/β-catenin signaling pathway is deregulated in most colon cancers as a result of mutation of APC or β-catenin (*CTNNB1*) genes. In several human colon cancer cell lines analyzed, 1α,25$(OH)_2D_3$ repressed β-catenin/TCF-4 transcriptional activity and thus inhibited the expression of β-catenin/TCF-4-responsive genes. Using oligonucleotide microarrays, the genetic profile induced by 1α,25$(OH)_2D_3$ in human colon cancer cells was identified. 1α,25$(OH)_2D_3$ changed the expression levels of numerous previously unreported genes, including many involved in transcription, cell adhesion, DNA synthesis, apoptosis and intracellular signaling. Vitamin D receptor (VDR) is expressed in normal colon epithelium and during the early stages of colon cancer, but is lost at later stages of tumor progression. High VDR expression has been associated with good prognosis. We found that the SNAIL transcription factor represses human *VDR* gene expression in colon cancer cells and blocks the antitumor action of EB1089 in xenografted mice. In human colon cancer, elevated SNAIL expression correlates with the down-regulation of *VDR* and E-cadherin. Our data predict that colon cancer patients with high levels of SNAIL are likely to be poor responders to therapy with 1α,25$(OH)_2D_3$ analogs.

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HIGH-DOSE PULSE CALCITRIOL IN PROSTATE CANCER

Anne Myrthue and Tomasz M. Beer

Oregon Health and Science University Portland, OR, U.S.A.

In pre-clinical models of prostate cancer, calcitriol, the principal active metabolite of vitamin D, displayed significant antineoplastic activity alone and in combination with cytotoxic drugs, but only at substantially supraphysiologic concentrations. The reported mechanisms of activity include inhibition of proliferation and cell cycle

PROGRAM & ABSTRACTS

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triggered meiosis? To address these issues, we have further characterized the steroid binding and signaling properties of the classical Xenopus progesterone and androgen receptors. We demonstrate that the Xenopus progesterone receptor (XPR-1) binds to and is transcriptionally activated by many androgens, including androstenedione, testosterone, and dihydrotestosterone, at 100 - 200 nM concentrations. Furthermore, the Xenopus androgen receptor (XeAR) binds to and is activated by progesterone at even lower concentrations (1 - 20 nM). Interestingly, the traditional mammalian PR antagonist RU486 is a poor inhibitor of progesterone binding to XPR-1 (IC50>1 nM), but a potent inhibitor of androgen binding to XeAR (IC50=5 nM). The inability of RU486 to significantly block progesterone interactions with XPR-1 is likely due to a cysteine at position 376 in the ligand binding domain, as most RU486-sensitive PRs have a glycine residue at this position. Accordingly, XeAR contains a glycine residue in the corresponding position, which might explain its sensitivity to RU486. Together, these results reconcile the discrepancies regarding which classical receptors are regulating progesterone and androgen-triggered *Xenopus laevis* oocyte maturation, as both receptors are likely activated under either condition, but RU486 and androgen receptor antagonists will only block XeAR-mediated effects.

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P2-657

Androgen Receptor Down-Regulation by the Antiestrogen ICI 182,780 (Faslodex) in LNCaP Prostate Cancer Cells.

Rumi S Bhattacharyya*[1], Aruna V Krishnan[1], Srilatha Swami[1], David Feldman[1]. [1]*Dept of Med, Endocrinol Div, Stanford Univ Sch of Med, Stanford, CA.*

For prostate cancer (PCa) patients not cured by primary therapy, androgen deprivation therapy (ADT) is often successful in causing PCa regression since these cancers are generally androgen-dependent. Unfortunately, most men eventually fail ADT and their disease transforms to an androgen-independent PCa (AIPC), for which there is no effective treatment. In AIPC, most PCa cells still retain expression of the androgen receptor (AR) which plays a role in the continued growth of the cancer. We hypothesize that drugs that are Selective AR Down-Regulators (SARDs) present a unique approach for the treatment of AR-dependent AIPC. Decreasing the AR concentration will significantly reduce PCa growth stimulation. Our studies have demonstrated that the antiestrogen, ICI 182,780 (ICI) (Faslodex) has SARD activity in LNCaP PCa cells. Treatment of LNCaP cells with ICI (10 μM) resulted in a 50% decrease in AR protein expression after 48 hrs as measured by [^{3}H]-DHT binding and Western blot analysis. ICI also decreased AR mRNA levels. Decreased AR mRNA expression was observed as early as 6 hrs after ICI treatment, the maximal decrease (60% of control) being seen at 24 hrs and by 48 hrs the inhibitory effect of ICI was diminished. Preliminary experiments aimed at understanding the mechanism of AR down-regulation by ICI suggest that it is occurring at the transcriptional level. The AR present in LNCaP cells contains a point mutation that renders it "promiscuous", allowing non-androgen ligands to bind to the receptor. In competition binding assays, increasing concentrations of ICI did not displace [^{3}H]-DHT binding to the AR, demonstrating that ICI did not bind to the mutant AR at the DHT binding site. AR down-regulation by ICI resulted in decreased AR-mediated functional responses as measured by PSA secretion and PSA mRNA expression. ICI inhibited R1881 stimulated PSA secretion by 60-70% after a 6 day treatment. PSA mRNA expression was decreased by 30-40% after 24 hrs of ICI and R1881 co-treatment when compared to R1881 alone. Importantly, ICI caused significant inhibition of LNCaP cell growth in a time and dose-dependent manner. At the end of 6 days of treatment a 60% growth inhibition was seen in ICI-treated cells compared to control. These data demonstrate that the anti-estrogen ICI is a potent AR down-regulator which causes significant inhibition of PCa cell growth. SARDs, such as ICI, present viable new options for treating AR-dependent advanced PCa.

P2-658 ⊗

Regulation of Prostaglandin Metabolism by Calcitriol: Potential Role in the Treatment of Prostate Cancer.

Jacqueline Moreno*[1], Aruna V Krishnan[1], David Feldman[1]. [1]*Dept of Med, Stanford Univ Sch of Med, Stanford, CA.*

Calcitriol exhibits growth inhibitory and pro-differentiation effects in *in vitro* and *in vivo* models of prostate cancer (PCa). Our goal is to define the mechanisms underlying the antiproliferative effects of calcitriol in PCa. cDNA microarray analysis of LNCaP human PCa cells showed the regulation of the expression of 28 genes by calcitriol. Interestingly, two of these genes are involved in the metabolism of prostaglandins (PGs), known stimulators of PCa cell growth. The expression of PG synthesizing cyclooxygenase-2 (COX-2) gene was significantly decreased by calcitriol, and that of PG inactivating 15-prostaglandin dehydrogenase (15-PGDH) was increased by calcitriol. This dual action of calcitriol would reduce the levels of biologically active PGs in PCa cells, thereby decreasing their proliferative stimulus. In the current study we showed that calcitriol increased 15-PGDH mRNA and protein levels in LNCaP cells in a time and dose-dependent manner. The increase in 15-PGDH mRNA expression reached a peak after 6 h of calcitriol treatment that was maintained over 36 h. Calcitriol reduced COX-2 mRNA to approximately 50 % of control in both LNCaP and PC-3 cells and decreased the concentration of COX-2 protein in PC-3 cells. We observed a 60 % decrease in levels of PGs in the conditioned media of LNCaP cells with calcitriol. We believe this decline is the result of the dual effect of calcitriol on the expression of PG metabolic enzymes. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX-2 and exhibit antitumor effects in both *in vitro* and *in vivo* PCa models. As a down-regulator of COX-2 expression, calcitriol would enhance COX-2 inhibition when combined with an NSAID. We found that the combination of calcitriol with NSAIDs acted synergistically to reduce the growth of LNCaP and PC-3 cells, exhibiting up to 50% more growth inhibition with the combination than induced by NSAIDs or calcitriol alone. The combination was also more effective than individual drugs in reducing PG secretion by LNCaP cells. The ability of calcitriol to inhibit PG synthesis and stimulate PG catabolism is an additional pathway by which calcitriol exerts its antiproliferative actions. The therapeutic combination of calcitriol and NSAIDs would allow the use of lower concentrations of either drug, reducing their toxic side-effects. We propose that calcitriol and NSAID combination might be a useful therapeutic strategy in men with early recurrent PCa.

P2-659

Direct Regulation of Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3) by Androgens and Calcitriol in LNCaP Human Prostate Cancer Cells.

Lihong Peng*[1], Jining Wang[1], Peter J Malloy[1], David Feldman[1]. [1]*Med, Stanford Univ Sch of Med, Stanford, CA.*

Calcitriol exhibits growth inhibition and differentiation in a variety of cell types including prostate cancer cells (PCa). In the androgen dependent LNCaP human PCa cell line calcitriol exerts its antiproliferative action predominantly by inducing cell cycle arrest. Previously we have shown that growth arrest is mediated primarily by induction of IGFBP-3, which subsequently increases the expression of the cell cycle inhibitor p21. We have identified a functional vitamin D response element (VDRE) in the IGFBP-3 promoter directly mediating the induction by calcitriol. In this report we show that androgens increase expression of IGFBP-3 at both mRNA and protein levels in LNCaP cells. Furthermore, the combination of calcitriol and androgens results in a substantial increase in IGFBP-3 indicating a strong synergistic effect of calcitriol and androgens on IGFBP-3 expression. To understand the molecular mechanism involved, we examined the IGFBP-3 promoter for interactions between calcitriol and androgens. Transactivation assays show that the 6 kb IGFBP-3 promoter sequence responds to androgen treatment. A time course of IGFBP-3 mRNA expression in LNCaP cells treated with various concentrations of the synthetic androgen R1881 also suggests that androgen directly regulate the transcription of IGFBP-3 in a dose-dependent manner. A series of deletions generated within the 6 kb promoter demonstrated that the ARE is present in the DNA fragment between -1753 and –3590. Point mutations in the potential ARE resulted in a loss of androgen induction confirming the critical response element sequences. Furthermore, chromatin immunoprecipitation assays showed that R1881 treatment recruited the androgen receptor to the ARE site in the IGFBP-3 promoter in intact cells. In addition, the combination treatment of androgens and calcitriol doubles the effect of either calcitriol or androgens alone on the IGFBP-3 promoter constructs. In conclusion, the functional VDRE and ARE in the IGFBP-3 promoter directly mediate the interaction of calcitriol and androgens on IGFBP-3 expression. It may be counter-intuitive that androgens stimulate a factor mediating antiproliferative and proapoptotic actions on PCa cells. However, a number of studies show that androgens mediate an antiproliferative and prodifferentiative action on PCa cells. The clinical significance of these findings will require further study.

P2-660 ♦

Co-Treatment of Human Prostate Cancer Cells with S179D Prolactin and 1,25 dihydroxy Vitamin D3 (VD) Produces Synergy in the Promotion of Apoptosis and Brings the Dose of VD Required into the Non-Toxic, Physiological Range.

Wei Wu[1], Amese M Walker*[1]. [1]*Biomed Scis, Univ of California, Riverside, CA.*

S179D prolactin (PRL) is a molecular mimic of phosphorylated human PRL that inhibits the growth of human prostate cancer cells when these are grown in vitro or as tumors in nude mice (1). In this study, we have investigated the interplay between S179D PRL and another inhibitor of prostate cancer growth, 1, 25 dihydroxy vitamin D3 (VD). When prostate cancer cells were incubated in S179D PRL for 3 days, doses up to 40 nM had no effect on cell number, although longer incubations were very effective at 10 nM. In the 3-day time frame, incubations up to 160 pM VD were also ineffective at reducing cell number, whereas those between 30 and 120 nM were effective. Incubation in S179D PRL at 20 nM (DU145 cells) or 10 nM (PC3 cells) sensitized the cells to VD such that a 50% reduction in cell number occurred at 60 pM and 100 pM VD in DU145 and PC3 cells, respectively. When DNA degradation was assessed, S179D PRL alone (same concentrations) and VD alone (100 pM) had no effect, but together there was marked DNA degradation. The 3-day incubation in either S179D PRL or VD produced no significant increase in the amount of the VD receptor (VDR), whereas co-incubation in S179D PRL and VD doubled the amount of the VDR, as assessed by Western blot. The same incubation conditions resulted in a doubling of p21 protein in response to S179D

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P3-60

Regulation of the Prostaglandin Pathway by Calcitriol in Prostate Cancer.

Jacqueline Moreno*[1], Lihong Peng[1], Srilatha Swami[1], Aruna V Krishnan[1], Candice S Johnson[2], David Feldman[1]. [1]*Dept of Med, Stanford Univ Sch of Med, Stanford, CA;* [2]*Dept of Med, Roswell Park Cancer Inst, Buffalo, NY.*

Calcitriol exhibits anti-proliferative and pro-differentiation effects in prostate cancer (PCa). Our goal is to identify calcitriol target genes mediating these actions. We have recently shown that calcitriol regulates genes involved in the metabolism of prostaglandins (PGs), known stimulators of cancer growth. We found that calcitriol inhibits PG actions in PCa cells by three mechanisms: stimulating the expression of the PG catabolizing enzyme 15-prostaglandin dehydrogenase (PGDH), decreasing the expression of the PG synthesizing enzyme cyclooxygenase-2 (COX-2) and inhibiting EP2 and FP PG receptor gene expression. The combination of calcitriol with non-steroidal anti-inflammatory drugs (NSAIDs) acted synergistically to achieve substantial PCa cell growth inhibition at ~2 to ~10 times lower concentrations of the drugs than when used alone. To gain insight into the mechanism by which calcitriol regulates the expression of PG-pathway genes, we examined the promoter sequences of both PGDH and COX-2 genes and identified putative vitamin D regulatory elements (VDREs) in each promoter by computer analysis. We cloned a 2.3 kb fragment of the human PGDH promoter into the luciferase reporter vector pGL3 basic and transiently transfected it into LNCaP cells. Preliminary results indicate a modest but significant induction of reporter luciferase activity in response to 10 nM calcitriol. Similar transfection assays with a 7.1-kb COX-2 promoter-luciferase construct in LNCaP DU-145 cells indicated that calcitriol significantly suppressed EGF-stimulated COX-2 promoter activity. These results suggest that calcitriol regulates the expression of the PGDH and COX-2 genes directly at the transcriptional level. To examine the effect of calcitriol on the regulation of the PG-axis *in vivo*, we established LNCaP xenografts in nude mice and treated them with calcitriol at a dose of 0.75 µg/mouse for three consecutive days. Analysis of mRNA expression in tumor samples revealed that calcitriol reduced the expression of COX-2 mRNA by 50%, indicating the regulation of the PG pathway by calcitriol *in vivo*. Further *in vivo* studies investigating the effects of calcitriol, NSAIDs and their combination are in progress. We believe that these studies will generate pre-clinical data that will lead to a clinical trial to test the combination therapy of calcitriol and NSAIDs in PCa patients.

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